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13. ABSTRACT *(Maximum 200 Words)*

We are investigating the role of AP-1 in controlling breast cell growth and transformation. We proposed to determine the role of the AP-1 family of transcription factors in mediating peptide growth factor-induced proliferation and oncogene-induced transformation of breast cells. Previous results demonstrated that AP-1 complexes are activated by peptide and steroid growth factors in normal and malignant breast cells, and that normal breast cells express higher levels of AP-1 proteins and activity than do breast cancer cells. We also previously showed that normal and immortal cells are more dependent on AP-1 for their growth than are most breast cancer cells. Over the last year, we determined whether AP-1 activation is required to transduce growth factor-induced signals in breast cancer cells. To perform these studies, we isolated MCF7 and MDA MB 435 clones that express a dominant-negative cJun mutant (TAM-67) under the control of an inducible promoter. These studies demonstrated that MCF7 cells, but not MDA MB 435 cells, depend on AP-1 for growth in serum. We also present results showing that inhibition of AP-1 completely blocked MCF7 proliferation induced by IGF-1 and EGF, yet only partially inhibited growth induced by estrogen. These results demonstrate that the mitogenic pathways in MCF7 cells activated by serum, estrogen, IGF-1, and EGF depend on AP-1 to transduce a proliferative signal, and that estrogen partially overcomes the growth suppressive effect of AP-1 blockade. These results suggest that AP-1 is a promising target of future cancer therapeutic and preventive agents since blocking this critical transcription factor suppresses proliferation induced by multiple growth factors.

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INTRODUCTION

During the third year of the funding period we have continued to investigate the affect of AP-1 blockade on the growth of normal, immortal, and fully malignant breast cells. These studies have demonstrated that the growth of normal, immortal and some malignant breast cells is suppressed by AP-1 blockade, while the growth of other breast cancer cells is not suppressed. We have also generated breast cancer cell lines that express the AP-1 inhibitor, TAM67, under inducible conditions. The growth of MCF-7 cells was sensitive to AP-1 blockade, while the growth of MDA MB 435 cells was not, consistent with our previous results. We have also discovered that AP-1 activation is required to transduce growth factor-induced mitogenic signals in breast cells. These studies showed that inhibition of AP-1 completely blocked proliferation of MCF7 cells in response to the peptide growth factors (IGF-1 and EGF), while inhibition of AP-1 slowed the growth of estrogen-stimulated cells, but did not completely suppress proliferation. These results demonstrate that the mitogenic pathways activated by serum, IGF-1, and EGF depend on AP-1 to transduce a proliferative signal, and that the proliferation induced by estrogen is at least in part, independent of AP-1. In the next year, we will utilize these reagents to investigate the role of AP-1 transcriptional activity in oncogene-induced transformation of breast cells.

These studies have demonstrated an involvement of AP-1 transcription complexes in regulating human breast cell proliferation at different stages of the transformation process. The results from these studies will provide the foundation for future efforts to develop agents that interfere with AP-1 signaling pathways. Such agents will likely be useful chemopreventive agents to block breast carcinogenesis.

BODY

BACKGROUND

Breast cancer is the most common malignancy in women, and the leading cause of death for women between the ages of 40 and 55 in this country (1). Even with aggressive mammographic screening, adjuvant chemotherapy, and intensive therapy for existing cancer, many of the women who develop breast cancer will die from it. Thus, more effective prevention strategies and treatments are urgently needed.

Unfortunately, little is known about the specific molecular events, which cause the progressive transformation of human breast epithelial cells to malignant breast cancer. Studies of model systems of cancer have revealed that multiple steps are involved in carcinogenesis, including tumor "initiation" and "promotion" events (2). Mutations and deletions within tumor suppressor genes may represent the molecular equivalent of breast cancer "initiation" events (3,4). However, the molecular mechanism of breast tumor "promotion" is poorly defined. In model systems (5), classic tumor promoters induce the proliferation of initiated cells, leading to the progressive outgrowth of fully malignant cells. Such tumor promoters typically activate signal transduction pathways to stimulate cellular proliferation. In human breast cells, the overproduction of growth factors, or aberrant stimulation of growth factor receptors, may be responsible for the promotional phase of breast carcinogenesis. Growth factors important for mammary epithelial cells, such as estrogen, EGF, TGFa, and the IGFs, may all represent tumor promoters of human breast cancer. Thus, drugs which inhibit the ability of estrogen to activate the estrogen receptor (tamoxifen and other antiestrogens) are used to treat breast cancer, and other drugs which block growth factor receptors, such as antibodies specific for the epidermal growth factor receptor and the Her2/neu receptor, have been shown to inhibit breast cancer cell proliferation (9-11) and are now being tested in clinical trials for the treatment of breast cancer. However, inhibition of individual signal transduction pathways may ultimately be ineffective, since multiple different signal transduction pathways can stimulate breast cell proliferation. It may be more effective to inhibit signal transduction at a more distal point in the cascade, where multiple mitogenic signals converge. Since transcription factors, the nuclear proteins that control DNA transcription and gene expression, are the most distal components of these converging mitogenic signal transduction pathways, they may be ideal targets for new therapeutic agents.

A key family of transcription factors transducing multiple mitogenic signals is the AP-1 family. These transcription factors are complexes of DNA-binding proteins made up of dimers of Jun and Fos proteins, which bind DNA at specific AP-1 sites and regulate the transcription of AP-1-dependent genes. AP-1 transcription factors are expressed in most cell types, and are activated by specific kinases, such as the mitogen-activated and stress-activated kinases, which are themselves activated by diverse signals such as growth factor stimulation, exposure to UV light, oxidative stress, tumor promoters such

as TPA, or oncogene overexpression or activation (reviewed in 6). Thus, AP-1 is a central component of many signal transduction pathways in a variety of cell types.

Previous studies showed that the AP-1 transcription factor family is critical for growth factor induced proliferation of fibroblasts (12,13). In addition, we (14,15) and others (16) have shown that AP-1 is also critical for oncogene-induced transformation of fibroblasts. Specifically, we have demonstrated that AP-1 is critical for the cotransformation of primary rat embryo cells by *ras+jun*, *ras+fos*, or *ras+SV40 T antigen* (14), while others have shown that AP-1 is critical for the transformation of NIH3T3 cells by single oncogenes such as *ras*, *raf*, *abl*, and *mos* (16). Thus, AP-1 is a central regulator of transformation as well as mitogenic signaling.

While the role of AP-1 has been extensively studied in fibroblasts, relatively few studies of the function of AP-1 have been performed in epithelial cells. Thus the exact role of this transcription factor family in controlling the proliferation and transformation of epithelial cells is not known. Previous studies from our lab and others have demonstrated that the Jun and Fos family members are expressed in human breast cancer cells, and are activated by a variety of important growth factors for these cells, such as EGF, TGFa, and the IGFs. Other studies have also suggested that hormones such as estrogens and retinoids can modulate AP-1 transcriptional activity in breast cells. More recent studies suggest that ER and AP-1 interact to regulate the expression of certain estrogen and/or tamoxifen regulated genes (17). AP-1 complexes may be involved in regulating transcription of the ER gene as well (18). These results suggest that the AP-1 complex may be involved in controlling proliferation of human breast cells. However, definitive studies demonstrating that AP-1 is critical for either breast epithelial cell proliferation or transformation have not been performed.

To address these questions, we are using the 184 series of normal human mammary epithelial cells (HMECs) isolated and characterized by Dr. Martha Stampfer (19). These cells were originally isolated from reduction mammoplasties of patients and have a normal karyotype, EGF receptors, and specific cytokeratins, suggesting that they are derived from the basal epithelial cells of the normal breast. These HMECs are primary cells, which will senesce after 15-20 passages. However, by exposing these primary HMECs to the carcinogen benzo(a)pyrene, Stampfer *et al.* (19) have established multiple immortalized lines of HMECs (the 184A1 and 184B5 lines). We are studying these carcinogen-immortalized cells as well as the spontaneously immortalized HMEC line, MCF10A, derived from breast tissue obtained from a patient with multiple fibrocystic nodules (20). This cell line expresses cytokeratins and epithelial mucins consistent with a breast epithelial origin, and has cytologic characteristics of breast luminal ductal cells (21). None of the immortal cells are fully transformed since they are not able to grow in an anchorage-independent fashion, or form tumors in nude mice. Recent reports have demonstrated that these immortalized human mammary epithelial cells can be transformed by specific oncogenes such as activated *ras* (22, 23) or *erbB2* genes (24), or by overexpression of *c-myc* or *SV40 T* genes (16). In particular, MCF10A cells can be transformed by an activated *ras* gene (23), while 184B5 cells can be fully transformed by activated *ras* genes (22), or by

overexpression of c-*erbB2* (24). Many of these oncogenes are known to activate AP-1 in fibroblasts, though whether these oncogenes also activate AP-1 in breast epithelial cells is not yet known. If AP-1 is involved in regulating these processes, it might therefore serve as a target for the prevention or treatment of breast cancer. To determine the role of AP-1 in controlling breast cell growth and transformation, we proposed to test the following hypotheses:

1. Human breast epithelial cells at different stages in the carcinogenesis pathway express different levels of the AP-1 transcription factor.
2. Breast epithelial cells at these different stages have different requirements for AP-1 for their growth.
3. AP-1 transcription factor activity is necessary for *in vitro* transformation of human breast epithelial cells.

In our previous reports we demonstrated that AP-1 transcription factor expression and transcriptional activity is high in normal mammary epithelial cells and is progressively reduced as breast cells proceed towards malignancy. These studies were originally proposed in **Specific Aim 1**. We also previously reported data demonstrating that the growth of normal and immortal cells is suppressed by AP-1 blockade. These studies, proposed in **Specific Aim 2** of the original grant proposal, demonstrated that normal, immortal and some cancer cells depend on AP-1 to transduce mitogenic signals, and that normal cells are more sensitive to the AP-1 blockade than are breast cancer cells. We now report the results from our experiments performed over the previous year (the third year of this project). Studies described in this annual report demonstrate that the mitogenic pathways in MCF7 breast cancer cells activated by serum, IGF-1, and EGF depend on AP-1 to transduce a proliferative signal. We also present data showing that growth of breast cells induced by estrogen is only partially inhibited by AP-1 blockade. Such results suggest that mitogenesis induced by peptide growth factors is critically dependent on AP-1, while growth induced by estrogen is likely mediated partly through an AP-1-independent signal transduction pathway. These results are also described in the attached manuscript, now under review for publication in *Cancer Research*. We are now conducting experiments to investigate whether AP-1 blockade suppresses the transformation of breast cells as described in **Specific Aim 3**. These studies are presently ongoing.

EXPERIMENTAL METHODS AND PROCEDURES

Primary Cell Cultures and Cell Lines:
Human mammary epithelial cells and cell lines used in these studies (Table 1) include normal HMECs isolated from epithelial organoids of human breast from Clonetics (passages 9-10); normal 184 cells (15); 184A1 and 184B5, nontumorigenic immortal cell lines derived from benzo(a)pyrene-treated 184 cells (19); MCF10A (from Dr. J. Russo), a nontumorigenic spontaneously immortalized HMEC cell line; MCF10AneoT (from Dr. J. Russo, Fox Chase Cancer Center, Philadelphia, PA), a transformed cell line derived from MCF10A transfected by c-Ha-ras; MCF7 WT (wild-

type), a human breast adenocarcinoma cell line; MCF7 Adria, a doxorubicin (Adriamycin)-resistant subclone of MCF7 WT (from Dr. K. Cowan, National Cancer Institute, Bethesda, MD). Cells were grown in the following culture media: MEGM (Clonetics, San Diego, CA) for normal HMECs 184, 184A1, and 184B5 (19,25); DME/F-12 with 5% horse serum and supplements as described (20, 23) for MCF10A and MCF10AneoT [with 400 μg/ml Geneticin (G418, Life Technologies, Inc., Gaithersburg, MD); and Improved MEM (high zinc option; Life Technologies, Inc.) supplemented with 10% FCS and penicillin/streptomycin for MCF7 and MDA MB 435.

Transfection of Breast Cells:
184, clone 91, 184B5, MDA MB 231, MCF7, and T47-D breast cells were transfected using Fugene 6 reagent (Boehringer Mannheim) while MCF10A and MDA MB 435 breast cells were transfected using the LT-1 transfection reagent (PanVera Corp.) according to manufacturer's recommendations.

Western Analysis:
Equal amounts of total cellular protein extract were electrophoresed on a 12% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad). Primary antibody used was rabbit anti-cJun Ab-1 from Oncogene Science (Cambridge, MA). Blots were developed using the enhanced chemiluminescence (ECL) procedure (Amersham).

Luciferase Assay to Measure AP-1 Activity:
AP-1 transcriptional activating activity in cells was measured using the enhanced luciferase assay (Tropix) as previously described (27). The cells were transfected with the Col-Z-Luc reporter gene containing the luciferase gene linked to 1100 bp of the human collagenase gene promoter which contains a single AP-1 binding site (TGAG/CTCA) between nt. –73 and –60. Transfected cells were lysed 36 hours after transfection and Luciferase activity was measured with equal amounts of cell extract.

Cell Growth Assays:
Cell proliferation assay of stably transfected Tet-On and Tet-Off cell lines
The CellTiter 96^{TM} AQueous Non-Radioactive Cell Proliferation Assay (MTS assay; Promega, Madison, WI) was used to measure breast cancer cell growth according to the protocol provided by the manufacturer. 1000 to 2000 cells were seeded in a 96 well plate and doxycycline was added (MDA MB435 rtTA-vector or -TAM67 lines) or removed (MCF7 tTA-vector or -TAM67 lines) was added the next day and replaced every other day. A solution containing a 20:1 ratio of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium) and PMS (phenazine methosulfate) was added to the cells for 2 hours at 37° C and absorption at 495 nm was determined. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Cell proliferation assay of breast cells treated with specific growth factors:
The MTS assay described above was used to measure breast cancer cell growth after stimulation with specific growth factors, including serum (0 to 10%), EGF (0 to 100ng/ml), IGF-1 (0 to 100ng/ml), and estrogen (0 to 10^{-9} M). The cells were seeded in 24 well plates in full medium with doxcline (MCF7 cells) or without doxycycline (MDAMB435 cells). The cells were allowed to attach overnight, and then were washed and cultured in serum free medium (and in the case of estrogen treated cells, estrogen- and phenol red-free medium) for 48 hours. The media was then changed to doxycline-free media (MCF7 cells) or doxcycline-containing media (MDAMB435 cells), to induce TAM-67 protein, with different levels of the specific growth factors. The cells were then cultured at 37º C for 1 to 7 days. Cells were harvested every other day and the MTS assay was done as described above to measure proliferation. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

RESULTS

AP-1 Expression and Activity in Breast Cells:

In **Specific Aim 1** we proposed to determine whether changes in AP-1 expression or activity occur as breast cells progress through different stages of carcinogenesis. Breast cells used in this study are listed in Table 1. We have previously shown that normal human mammary epithelial cells have high basal AP-1 activity, immortal breast cells have an intermediate level of basal AP-1 activity, and breast cancer cells have low basal AP-1 activity. We described these results in the 1996-97 annual report and in a 1997 *Cancer Research* publication (Smith *et al.* 28).

Table 1: Breast cells used in this study.

Cells	Name	Source	Phenotype
Normal HMECs:	HMEC-91	Clonetics	Senescent, anchorage-dependent
	184	M. Stampfer	
Immortal HMECs:	184B5	M. Stampfer	Immortal, anchorage dependent
	MCF10A	A. Russo	
Breast Cancer cell lines:	MCF7 WT	K. Cowan	Cancer cells, anchorage-independent
	T47-D	ATCC	and tumorigenic
	MDA MB 231	ATCC	
	MDA MB 435	ATCC	

In **Specific Aim 2** we proposed to determine whether the growth of breast cells at different stages of tumorigenesis are differentially affected by inhibiting AP-1 activity. We have investigated the affect of AP-1 blockade on the growth of normal, immortal, and fully malignant breast cells. These studies have demonstrated that the growth of normal and immortal and some malignant cells is suppressed by AP-1 blockade, while the growth of some other breast cancer cells is not suppressed. These results were described in the 1997-98 annual report and in the submitted manuscript by Ludes-

Meyers *et al.*, which is now under review for publication in *Cancer Research* (and is included in the Appendix).

Tet-On And Tet-Off Inducible Breast Cancer Cells Expressing TAM-67:

We previously described the isolation of breast cancer cell lines that express TAM-67 under the control of Tet-inducible or Tet-repressible promoters. These breast cancer cell lines were used in the studies described in this report. For these experiments, we used the following cells:

   a) **MCF7-Tet-Off TAM-67 cells**: These cells do not express TAM-67 when the cells are grown in doxycycline (1ug/ml), but do express TAM-67 protein when grown in the absence of doxycyline (see Figure 1a).

   b) **MDA MB 435 Tet-On TAM-67 cells**: These cells do not express TAM-67 when the cells are grown in the absence of doxycycline, but do express TAM-67 protein when grown in the presence of doxycyline (1ug/ml) (see Figure 1b).

Effect of TAM-67 Expression on Breast Cancer Cell Growth:

In the previous year's annual report, we presented the results of our preliminary studies of the Tet-On and Tet-Off cells. In this report, we now present our complete results. These studies now show that the growth of MCF-7 cells is inhibited when TAM-67 is induced and AP-1 activity is blocked (see Figure 2A). Three separate clones of MCF-7 cells were used in these studies. In each clone, the level of expression of TAM-67 protein correlates with the degree of growth inhibition. In MDA MB435 cells, blockade of AP-1 by TAM-67 does not affect the proliferation of these cells (see Figure 2B). Thus, in MCF7 cells (as in normal and immortal cells as shown in the previous year's annual report), AP-1 is necessary for proliferation in full serum. These results also suggest that AP-1 is not necessary for the growth of MDA MB435 cells in serum.

Effect Of Inhibiting AP-1 Transactivating Activity On Proliferation Induced By Specific Growth Factors:

Over the last year, we have investigated the effect of blocking AP-1 on mitogenesis induced by specific growth factors. We previously demonstrated that induction of TAM-67 protein is associated with inhibition of AP-1 transactivating activity. We next investigated whether induction of TAM-67 interfered with serum-induced proliferation of MCF7 Tet-Off TAM-67 cells. In these experiments, MCF7 Tet-Off TAM-67 cells (Clone #67), or MCF7 Tet-Off vector cells (Vector Clone #1) were treated with different concentrations of serum (0.5%, 1.0%, 5.0%) in the presence and absence of doxycyline (Figure 3). When the TAM-67 MCF7-Tet-Off clone was grown in the presence of doxycyline (no TAM-67 expressed), these MCF-7 clones proliferated normally in response to serum stimulation. However, when doxycyline was withdrawn, TAM-67 was expressed, and serum-induced proliferation was inhibited.

We next investigated whether the mitogenic response to specific growth factors also requires AP-1. MCF-7 Tet-Off cells were grown in the presence of IGF-1, EGF or estrogen, under uninduced and induced conditions, and proliferation was measured. As shown in Fig. 4, when the cells were grown in the presence of different concentrations of IGF-1 (0, 1, 10, 100 ng/ml), and in the presence of doxycyline, the MCF7-Tet-Off-TAM67 cells grew normally. When doxycyline was withdrown, TAM67 was expressed, proliferation response to IGF-1 was completely blocked. The control cells, MCF7-Tet-Off-vector grew normally in the presence or absence of doxycyline. Similar results were obtained when the cells were treated with increasing concentrations of EGF (0, 1, 10, 100 ng/ml) (Fig. 5).

We next studied the proliferative response to different concentrations of estrogen (0, 10^{-11}, 10^{-10}, 10^{-9} M). When doxycyline was added, TAM67 expression was suppressed, and MCF7-Tet-Off-TAM67 cells grew normally. When the doxycyline was withdrawn, TAM67 was expressed, and cell growth was suppressed but not completely blocked (Fig. 6). This partial suppression of growth is in contrast to the total blockade of proliferation induced by IGF and EGF. The implications of this difference between IGF-, EGF- induced growth as compared to estrogen-induced growth are discussed below.

Suppression of oncogene-induced transformation by AP-1 blockade:

In **Specific Aim 3** we proposed to determine whether inhibition of AP-1 activity prevents the *in vitro* transformation of immortalized breast cells. We have previously successfully transfected oncogenes, such as c-Ha-ras or c-erbB2, into immortal 184B5 and MCF10A cells and transformed these cells into cells that exhibit anchorage-independent growth. We are now using two different approaches to investigate whether TAM-67 blocks oncogene-induced transformation:

1). We are attempting reverse the phenotype of already transformed cells. For these experiments, we are transfecting TAM-67 into the Ras- and erbB2- trannsfected 184B5 and MCF10A cells. We will then measure the effect of AP-1 blockade on the anchorage independent growth of these cells.
2). We will attempt to block *de novo* transformation of 184B5 and MCF10A cells. For these experiments we will co-transfect oncogenes (either ras or erbB2) and TAM-67 (or vector as a control) into the parental 184B5 and MCF10A cells. To ensure that we obtain stable clones that express both genes, we will use an IRES vector system to deliver both genes at once. We will then measure AP-1 activity in these clones to ensure we have AP-1 blockade and then compare anchorage growth of vector and TAM-67 transfected cells.

From these experiments, we can determine whether AP-1 blockade prevents or reverses the transformed phenotype of oncogene-transformed cells.

If these studies demonstrate that TAM-67 blocks anchorage-independent growth, then in future studies we will investigate the effect of AP-1 blockade on *in vivo* tumorigenesis.

DISCUSSION

The data presented here, along with our previous data, demonstrate that AP-1 blockade induced by TAM-67 inhibits the growth of normal, immortal and some breast cancer cells (such as MCF7 cells), but that other breast cancer cells (such as MDA MB 435) are relatively resistant to AP-1 blockade. The present results also show that mitogenesis induced by individual peptide growth factors, such as IGF-1 and EGF, can be completely blocked by expression of TAM-67, while mitogenesis induced by estrogen is only partially inhibited by AP-1 blockade. These data suggest that peptide growth factor-induced mitogenesis is critically dependent on AP-1 in MCF-7 cells. The data also suggest that estrogen-induced mitogenesis is only partially dependent on AP-1 and that estrogen-induced signaling likely involves other AP-1 independent pathways.

Multiple growth factors have been shown to stimulate the proliferation or differentiation of normal HMECs and breast cancer cells (15, 29,30). EGF stimulates the growth of normal HMECs (15) as well as breast cancer cells (29,30), and heregulin has been found to modulate the growth and differentiation of immortal HMECs (31). Other hormones that affect the growth of breast cancer cells include estrogen (32,33) and insulin-like growth factors (IGF-1 and IGF-2) (34), which induce proliferation, and retinoids, which inhibit proliferation and induce differentiation (35,36). Previous studies from our own lab have demonstrated AP-1 complexes are activated by important growth factors for breast cells, such as EGF, and IGFs (28). These previous results suggested that the AP-1 complexes might be involved in controlling proliferation of human breast cells. The current data now demonstrated that peptide growth factors stimulate breast cancer cell proliferation through the AP-1 signaling pathway. Thus, the AP-1 transcription factor is a critical signaling molecule in normal breast cells and in some breast cancer cells (i.e. MCF-7), but apparently not in other breast cancer cells (i.e. MDA MB 435).

The present results also demonstrate that estrogen-induced proliferation of MCF-7 cells was only partially inhibited by AP-1 blockade. These suggest that estrogen induces mitogenesis through several different signal pathways. One of these pathways is likely dependent on AP-1, while other mitogenic pathways induced by estrogen .are likely independent of AP-1 (see Figure 7). Estrogen – induced signaling potentially intersects with AP-1 dependent pathways at several points along the signaling cascade. Firstly, estrogen can bind the estrogen receptor and the ER protein can interact with the AP-1 complex either directly through protein-protein interaction, or indirectly through bridging proteins. Previous studies by Webb *et al.* (37) showing that AP-1-ER interaction can occur in some cells, support this possible mechanism. Secondly, estrogen can bind to ER and activate gene expression that directly or indirectly increases c-Jun and c-Fos expression or activity. Data showing that estrogen treatment

causes increased expression and activity of AP-1 components has been published by several groups (38-40). A more indirect pathway by which estrogen can activate AP-1 is through induction of expression of IGF family. Previous studies have shown that estrogen treatment of MCF7 cells causes increased IGF-II and IGFR-I, IGFR-II and IGFBPs production (41-44). This increased IGF signaling then would activate AP-1. However, our data showing that estrogen-induced growth is only partially suppressed by AP-1 blockade, suggest that there are also AP-1independent signaling pathways activated by estrogen treatment of breast cells. Previous studies have suggested that estrogens regulate the expression and function of c-Myc (45-47) and cyclin D1 and activate cyclin E-Cdk2 complexes (48-50), all of which maght be rate limiting for progression from G1 to S phase. These pathways may induced proliferation independently of AP-1. Such AP-1-independent pathways may account for the lack of total growth suppression of estrogen treated MCF7 cells in which AP-1 is inhibited.

An alternative explanation for the partial inhibition of estrogen-induced growth by AP-1 blockade is that estrogen-induced mitogenisis is more difficult to block and would require more stringent AP-1 blockade. However, in light of the very high level of TAM-67 induced in these cells, this explanation is unlikely.

We have previously demonstrated that normal human breast cells have high basal levels of AP-1 activity and that breast cancer cells express relatively low levels of AP-1 activity. We have also shown that AP-1 complexes are activated by peptide and steroid growth factors in normal and malignant breast cells. In this report, we now show that the mitogenic pathways activated by serum, IGF-1 and EGF depend on AP-1 to transduce proliferative signal, and that estrogen-induced growth is only partially inhibited by AP-1 blockade. These results suggest that AP-1 is a promising target of future cancer therapeutic and preventive agents since blocking this critical transcription factor suppresses proliferation induced by multiple growth factors. However, these results also suggest that AP-1 inhibitors would be most effective in combination with anti-estrogen such as tamoxifen, since AP-1 blockade does not completely suppress estrogen-induced growth of breast cancer cells.

ONGOING STUDIES

We are continuing our studies of AP-1 inhibition in immortalized HMECs and breast cancer cells. During the following year of the grant we will:

Determine whether AP-1 blockade inhibits the transformed-phenotype of breast cancer cells and oncogene-transformed HMECs.

We proposed to determine whether inhibition of AP-1 activity prevent the *in vitro* transformation of immortalized breast cells. In our previous annual reports, we have described successful transfecting oncogenes, such as c-Ha-ras or c-erbB2 into immortal 184B5 and MCF10A cells. These transfected cells have been transformed into cells that grow in soft agar, unlike the parental immortal breast cells. We are presently transfecting these oncogene-transformed breast cells with TAM67 to determine

whether AP-1 blockade reverses this transformed phenotype. We are also testing whether TAM67 blocks de novo transformation of breast cells. For these experiments, we developed pIRES constructs which expressed both TAM67 and oncogenes (activated c-Ha-ras or c-erbB-2).

From these experiments, we can finally determine whether AP-1 blockade prevents or reverses the transformed phenotype of oncogene-transformed cells.

If these studies show TAM67 inhibits anchorage independent growth, then in future studies, we will investigate the effect of AP-1 blockade on *in vivo* tumorigenesis. Such studies will provide the foundation for the development of future cancer preventive agents that target the AP-1 transcription factor.

KEY RESEARCH ACCOMPLISHMENTS AND REPORTABLE OUTCOMES

Specific Aim 1: To determine whether changes in AP-1 expression or activity occur as HMECs progress through different stages of carcinogenesis.
We have completed this specific aim and have completed tasks for months 1-36 listed in the statement of work.

Accomplishments:
- Our results demonstrated that Jun and Fos protein expression and AP-1 activity are high in normal human mammary epithelial cells, and are reduced as breast cells progress toward a more malignant phenotype

Reportable Outcomes:
The results of these studies were:
- described in our first year annual report,
- presented at the "Era of Hope" meeting in Washington, D.C., November, 1997,
- published as an abstract in the Era of Hope meeting booklet, 1997
- published in *Cancer Research* (28).

Specific Aim 2: To determine whether growth of HMECs at the different stages is differentially affected by inhibiting AP-1 activity.
The tasks for months 1-36 have been completed and summarized in our previous reports and in this report.

Accomplishments:
- These studies demonstrate that normal and immortal human mammary epithelial cells require AP-1 for their growth.
- We successfully established MCF7 Tet-Off-TAM67 and MDAMB435 Tet-On-TAM67 cell lines.
- We discovered that breast cancer cells, which have relatively low basal AP-1 transcriptional activity, are less sensitive to AP-1 blockade. Of the breast cancer

cells tested, MCF7 cells were the most sensitive to the growth suppressive effect of the AP-1 inhibitor.
- We also found that AP-1 blockade completely inhibits proliferation of breast cancer cells in response to peptide growth factors (IGF-1 and EGF), but only partially inhibits estrogen-induced proliferation.

Reportable Outcomes:
These results were:
- presented at the "Era of Hope" meeting in Washingtion, D.C., in November 1997 and published as an abstract in the meeting booklet
- presented at the the San Antonio Breast Cancer Symposium in 1997 and 1998, and published as an abstract in *Breast Cancer Research and Treatment* (51, 52).
- described in the manuscript submitted for publication in *Cancer Research*.

Specific Aim 3: To determine whether inhibition of AP-1 activity can prevent the *in vitro* transformation of immortalized HMECs.
The tasks for months 1-36 have been completed and summarized in our previous reports and in this report.

Accomplishments:
- We have developed an *in vitro* transformation assay and have isolated oncogene-transformed HMECs and determined their transformed phenotype.
- We have established several clones of 184B5 and MCF10A cells that stably express an activated erbB2 oncogene or an oncogenic ras protein. All of these clones exhibit the transformed phenotype of anchorage independent growth.
- These transformed HMECs and breast cancer cells will be used to determine whether inhibition of AP-1 transcriptional activity reverses the transformed phenotype of breast cells. We are now conducting experiments to investigate how TAM67 will affect the *de novo* transformation of immortalized breast cells (tasks for months 36-48)..

Reportable Outcomes:
- We have derived oncogene-transformed 184B5 and MCF10A cell lines

CONCLUSIONS

During the third year of the funding period we investigated the effect of AP-1 blockade on the growth of normal, immortal and cancerous breast cells. We successfully established MCF7 Tet-Off-TAM67 and MDAMB435 Tet-On-TAM67 cell lines. We used these cell lines to determine the effect of TAM-67 expression on breast cancer cell growth and found that AP-1 activity is inhibited and the growth of MCF7 cells is suppressed when TAM-67 is induced. Thus, AP-1 is necessary for proliferation for MCF7 cells. Similar experiments done in MDA MB 435 cells demonstrate that AP-1 is not necessary for the growth of MDAMB 435 cells. We then used the MCF7 cells to

determine whether AP-1 blockade affected the proliferation induced by serum, EGF, IGF-1 and estrogen. The results demonstrated that AP-1 blockade completely inhibits proliferation of breast cancer cells in response to serum and peptide growth factors (IGF-1 and EGF), but only partially inhibits estrogen-induced proliferation. These data suggest that peptide growth factor-induced mitogenesis is critically dependent on AP-1 in MCF-7 cells. The data also suggest that estrogen-induced mitogenesis is only partially dependent on AP-1. Therefore estrogen-induced signaling likely involves other pathways.

These studies have demonstrated an involvement of AP-1 transcription complexes in regulating human breast cancer cell proliferation through different signaling pathways. The results from these studies will provide the foundation for future efforts to develop agents that interfere with AP-1 signaling pathways. Such agents may be useful chemopreventive agents to block breast carcinogenesis.

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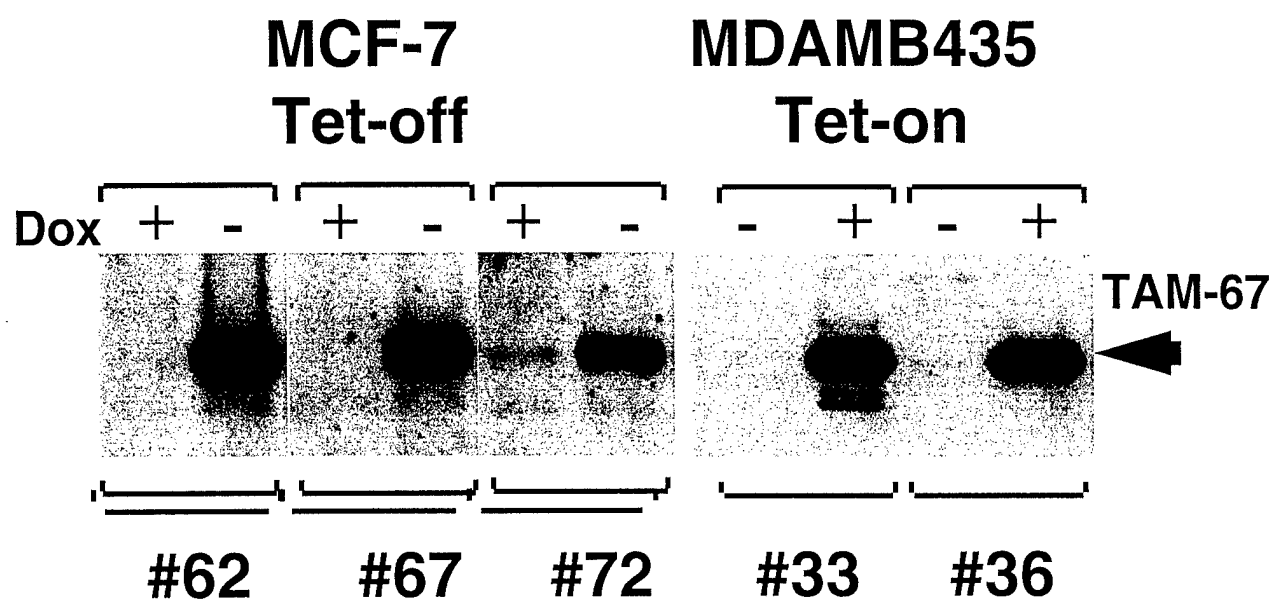
Figure 1. Immunodetection of Induced TAM67 Protein Expression in MCF7 Tet-Off (A) and MDA MB 35 Tet-On cells. Total cellular protein was extracted 48 hours after induction and equal amounts of protein was analyzed for TAM67 expression using anti-cJun antibody. The TAM 67 protein band is indicated with an arrow.

A.   MCF7 tet-off clones
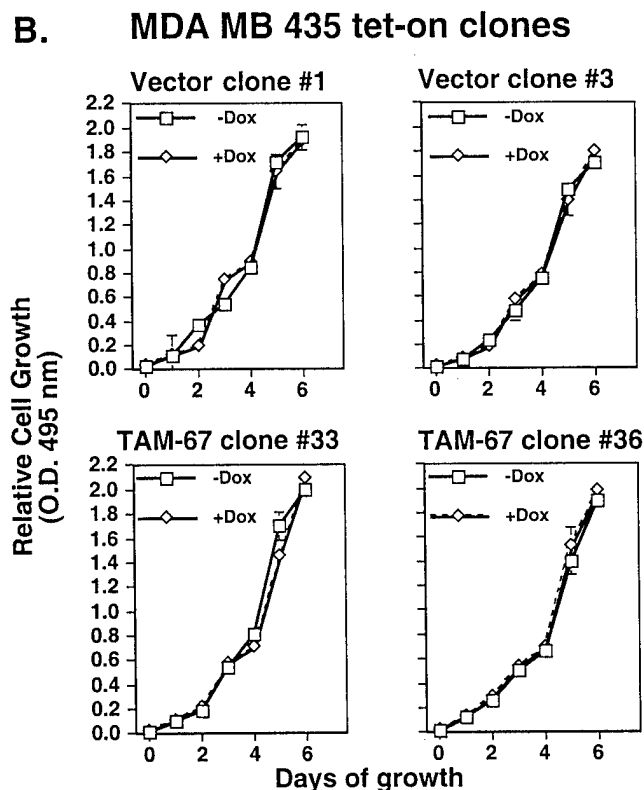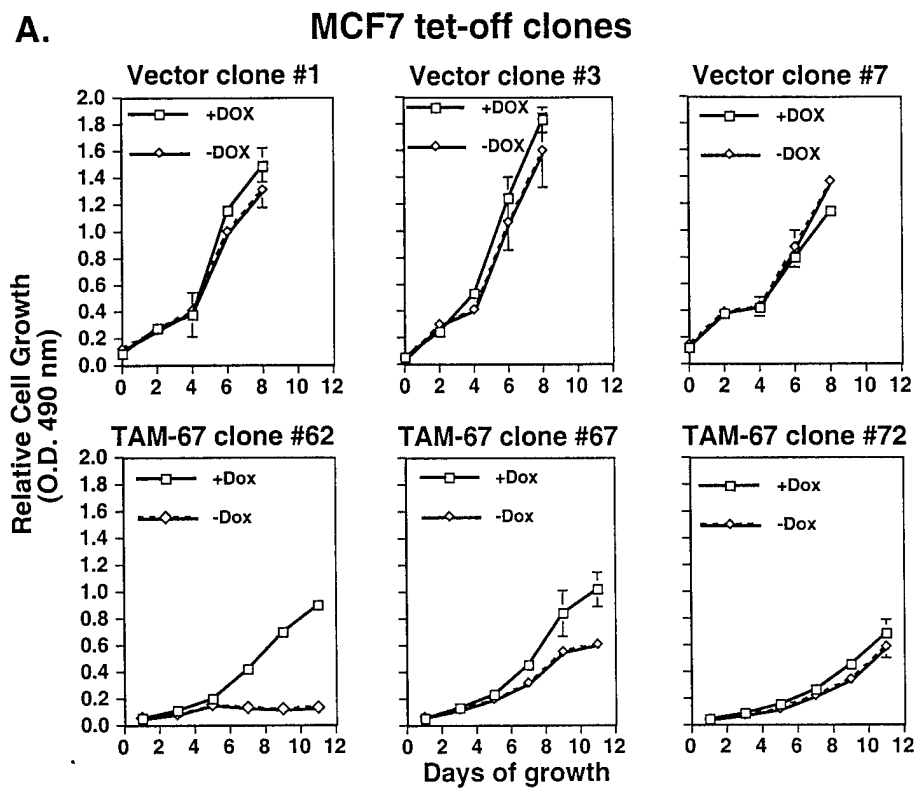
Figure 2. Effect of Induced TAM67 Expression on the Growth MCF7 and MDA MB 435 Breast Cancer Cells. Growth of MCF7 Tet-Off vector and TAM67 clones (A) and MDA MB 435 Tet-On vector and TAM67 clones (B) was determined in the presence and absence of 1ug/ml doxycyline. Every two days cell growth was measured as an increase in the O.D. 495 after incubation with medium containing a MTS/PMS substrate as described in " Experimental Methods and Procedures".
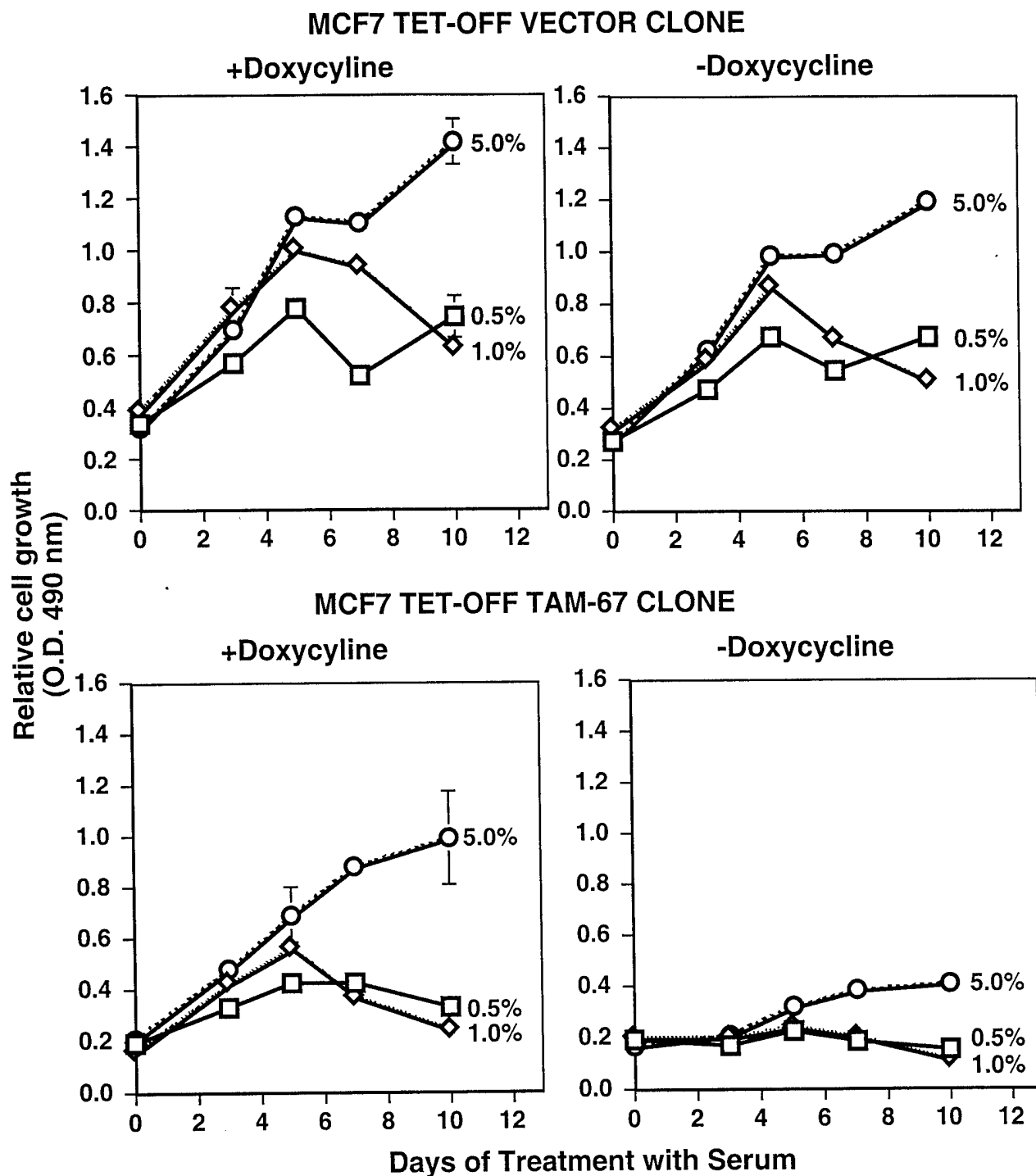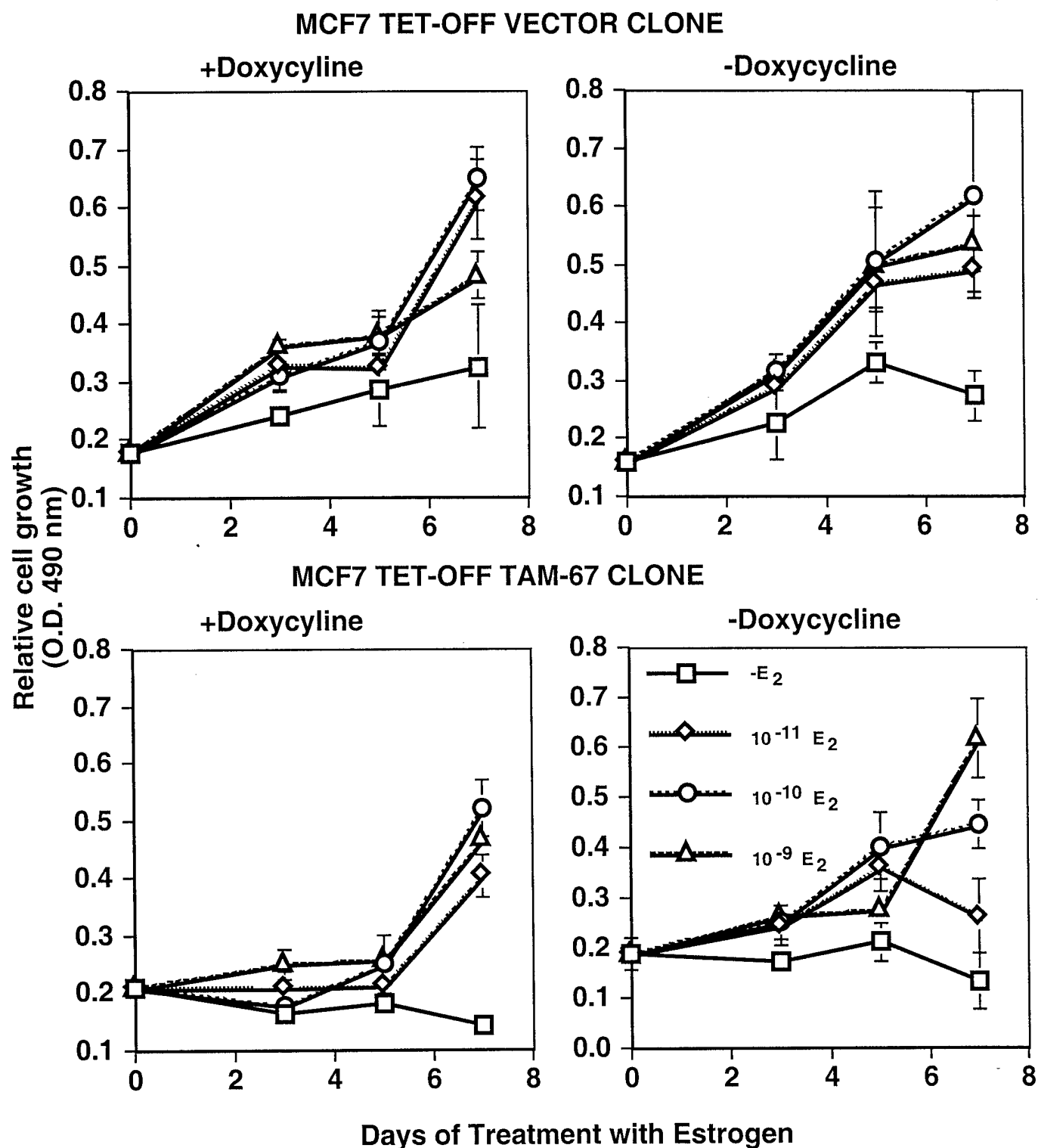
Figure 3.   AP-1 Blockade on the Proliferation of MCF7 Cells Induced by Serum. MCF-7 Tet-Off
TAM-67 and MCF-7 Tet-Off vector control cells, in the presence or absence of 1ug/ml doxycyline,
were cultured in the presence of 0.5%, 1.0% or 5.0% fatal bovine serum. Relative cell densities (O. D.
490 nm) were measured on the day serum treatment began (day 0) and every two days subsequent to the
addition of serum. Each data point was performed in quaduplet, and the results were reported as mean
absorption +/- standard error.
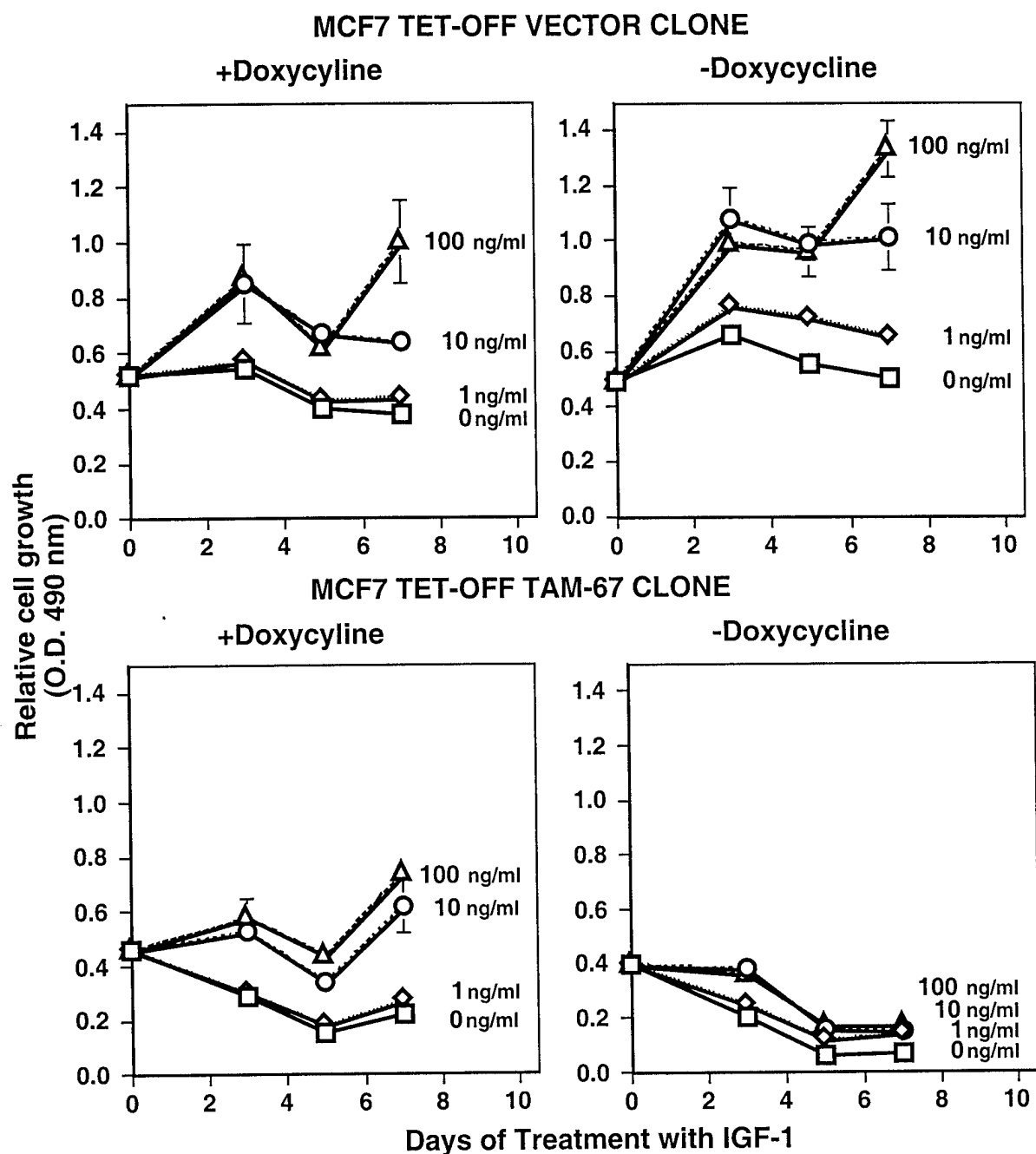
Figure 4. AP-1 Blockade on the Proliferation of MCF7 Cells Induced by IGF-1. MCF-7 Tet-Off
TAM-67 and MCF-7 Tet-Off vector control cells, in the presence or absence of 1ug/ml doxcline,
were cultured in the presence of 0, I, 10, 100ng/ml IGF-1. Relative cell densities (O. D. 490 nm) were
measured on the day estrogen treatment began (day 0) and every two days subsequent to the addition
of serum. Each data point was performed in quadruplet, and the results were reported as mean
absorption +/- standard error.
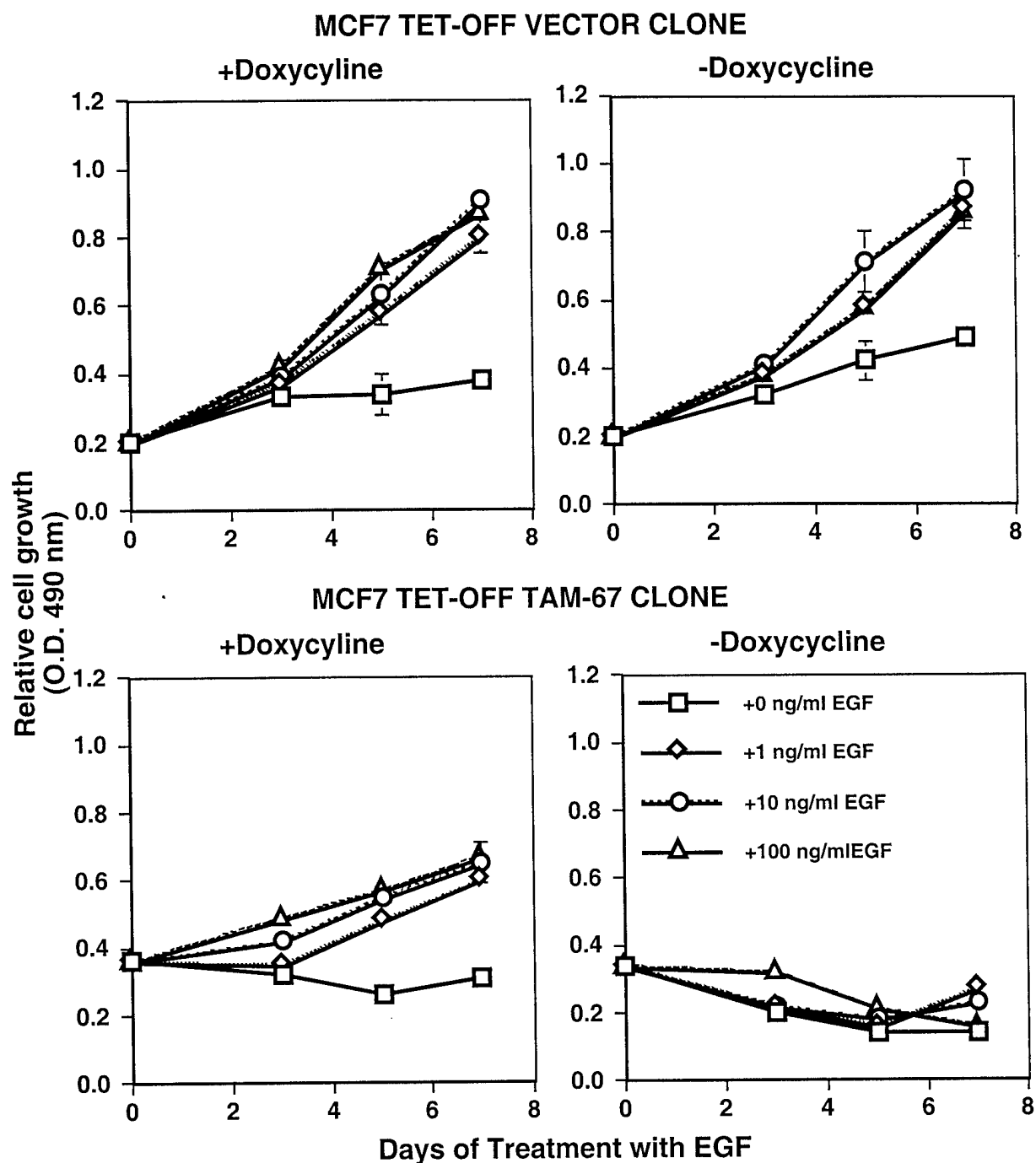
Figure 5. AP-1 Blockade on the Proliferation of MCF7 Cells Induced by EGF. MCF-7 Tet-Off TAM-67 and MCF-7 Tet-Off vector control cells, in the presence or absence of 1ug/ml doxycline, were cultured in the presence of 0, 1, 10, 100 ng/ml EGF. Relative cell densities (O. D. 490 nm) were measured on the day IGF-1 treatment began (day 0) and every two days subsequent to the addition of serum. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

MCF7 TET-OFF VECTOR CLONE

+Doxycyline

-Doxycycline

Relative cell growth (O.D. 490 nm)

MCF7 TET-OFF TAM-67 CLONE

+Doxycyline

-Doxycycline

Legend:
- $-E_2$
- $10^{-11}\ E_2$
- $10^{-10}\ E_2$
- $10^{-9}\ E_2$

Days of Treatment with Estrogen

Figure 6. AP-1 Blockade on the Proliferation of MCF7 Cells Induced by Estrogen. MCF-7 Tet-Off TAM-67 and MCF-7 Tet-Off vector control cells, in the presence or absence of 1ug/ml doxycyline, were cultured in the presence of no estrogen or 10^{-11} to 10^{-9} M estrogen. Relative cell densities (O. D. 490 nm) were measured on the day estrogen treatment began (day 0) and every two days subsequent to the addition of serum. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.
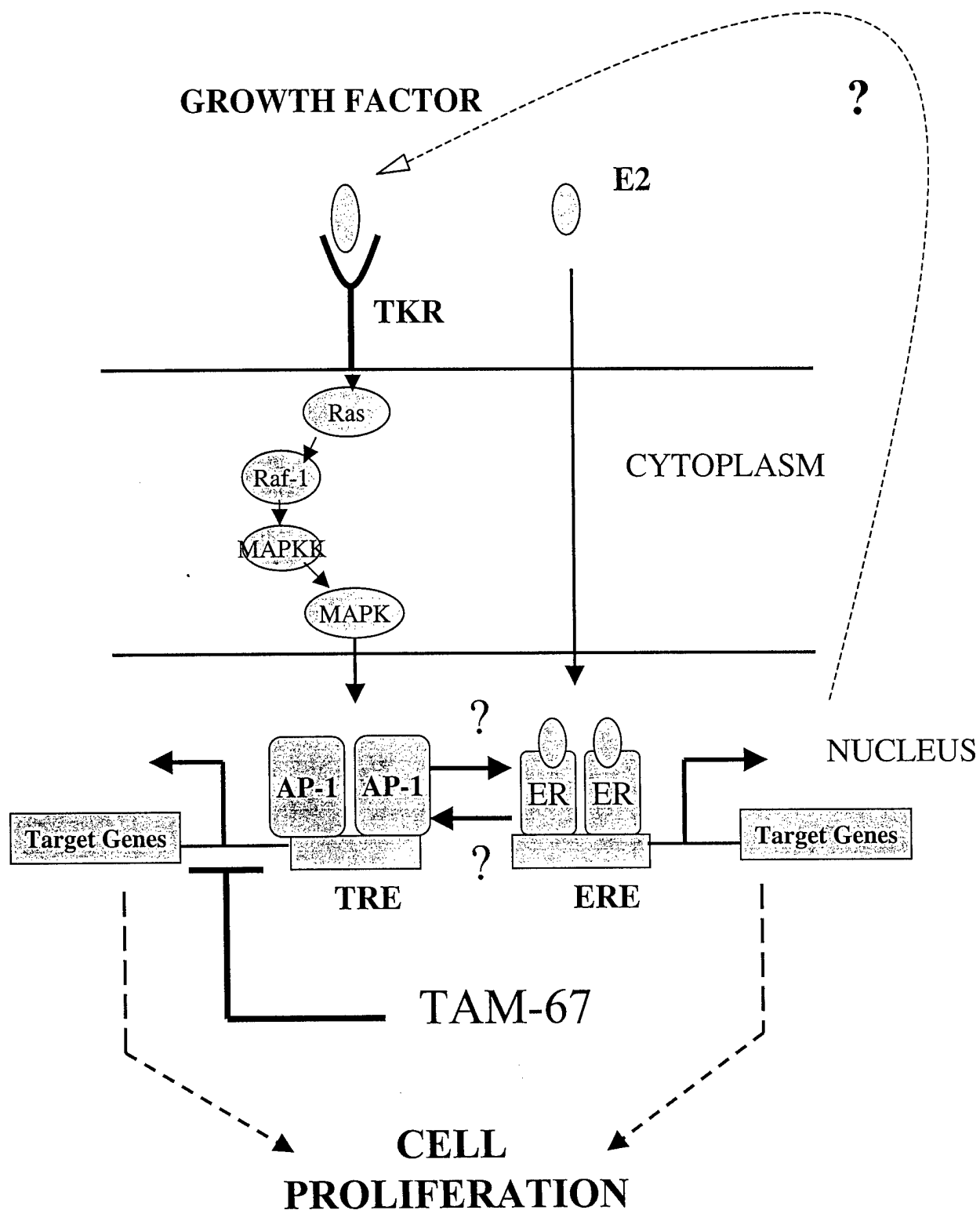
Fig. 7 A model of mitogenic pathways induced by growth factors and estrogen in breast cancer cells. ER: estrogen receptor; TKR: tyrosine kinase receptor; ERE: estrogen responsive element; TRE: TPA responsive element

ABBREVIATIONS

AP-1   Activating Protein 1
ATCC American Type Culture Collection
ATF   Activating Transcription Factor
bp   base pairs
CBP   Creb Binding Protein
cDNA complementary Deoxyribonucleic Acid
CMV Cytomegalovirus
DME Dulbecco's Modified Eagle
ECL   Enhanced Chemiluminesence
EGF   Epidermal Growth Factor
EGFR Epidermal Growth Factor Receptor
ER   Estrogen Receptor
ERE   estrogen responsive element
FCS   Fetal Calf Serum
Ha-ras Harvey-ras
HMEC Human Mammary Epithelial Cells
IGF   Insulin-like Growth Factor
IGFBP Insulin-like growth factor binding protein
MAPK mitogen activated protein kinase
MEGM Mammary Epithelial Growth Medium
MEM Modified Eagle Medium
ml   milliliter
MTS   3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-
     2-(4-sulfophenyl)-2H-tetrazolium
neo   neomycin transferase
nm   nanometer
O.D.   Optical Density
PMS   phenazine methosulfate
rtTA   reverse tetracycline-controlled transactivator
SEM   Standard Error of the Mean
SV40 Simian Virus 40
tTA   tetracycline-controlled transactivator
Tet   Tetracycline
TetRE Tetracycline Response Element
TGF   Transforming Growth Factor
TKR   tyrosine kinase receptor
TPA   12-O-tetradecanoyl-phorbol-13-acetate
TRE   TPA responsive element
ug   microgram
WT   Wild-type

Normal and Immortal Human Mammary Epithelial Cells are More Dependent on AP-1 for Their Growth Than are Breast Cancer Cells

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4. Abbreviations:
AP-1: Activating Protein-1; ER: Estrogen receptor; HMECs: Human mammary epithelial cells; IGF: insulin-like growth factor; SCPA: Single cell proliferation assay; SEM: standard error of the mean; TGFα: transforming growth factor alpha; TPA; 12-O-tetradecanoylphorbol-13-acetate; X-Gal: 5-Bromo-4-Chloro-3-Indoyl-ß-galactopyranoside

ABSTRACT

We have previously demonstrated that basal AP-1 transcriptional activity is high in normal human mammary epithelial cells, intermediate in immortal breast cells, and relatively low in breast cancer cells. In this study we investigated whether differences in AP-1 transcriptional activity reflect differences in breast cells' dependence on AP-1 for proliferation. The cJun dominant negative inhibitor, TAM-67, was used to determine the effect of AP-1 blockade on the growth of normal, immortal, and malignant breast cells. We first determined the effect of TAM-67 expression on colony forming efficiency of immortal and malignant breast cells. The AP-1 inhibitor reduced colony formation of immortal breast cells by over 50% (by 58% in 184B5 cells and 62% in MCF10A cells), and reduced colony formation of two breast cancer cell lines, MCF7 and MDA MB 435, by 43% and 17% respectively, but did not reduce colony formation in two other breast cancer cell lines (T47D, MDA MB231). We next determined the effect of AP-1 blockade on the growth of normal, immortalized and malignant breast cells using a single cell proliferation assay. Using this assay, normal breast cells were extremely sensitive to AP-1 blockade, immortal breast cells were moderately sensitive, and breast cancer cells were either modestly sensitive (MCF7 and MDA MB 435) or resistant (T47D and MDA MB 231). We next directly tested the effect of TAM-67 expression on the growth of MCF7 and MDA MB 435 breast cancer cells, using cells stably transfected with TAM-67 under the control of a doxycycline-inducible promoter. Upon induction, TAM-67 was expressed and AP-1 activity was inhibited in these cells. This AP-1 blockade suppressed the growth of MCF7 cells, however, AP-1 blockade did not affect the growth of MDA MB 435 cells. These results suggest that MCF7 cells are more sensitive to AP-1 blockade than are MDA MB 435 cells. These results demonstrate that normal breast cells are dependent on AP-1 to transduce proliferative signals, and that immortal breast cells and some breast cancer cells also require AP-1 to transduce these signals, but that other breast cancer cells may not require AP-1 for proliferation. These studies also demonstrate that breast cells' dependence on AP-1 diminishes as these cells progress to malignancy.

INTRODUCTION

Breast cancer is one of the most common malignancies in women, and the leading cause of death for women between the ages of 40 and 55 in this country (1). Even with aggressive mammographic screening, adjuvant chemotherapy, and intensive therapy for existing cancer, many of the women who develop breast cancer will die from it. Thus, more effective prevention strategies and treatments are urgently needed.

Unfortunately, little is known about the specific molecular events, which cause the progressive transformation of human breast epithelial cells to malignant breast cancer. Studies of model systems of cancer have revealed that multiple steps are involved in carcinogenesis, including tumor "initiation" and "promotion" events (2). Mutations and deletions within tumor suppressor genes may represent the molecular equivalent of breast cancer "initiation" events (3, 4). However, the molecular mechanism of breast tumor "promotion" is poorly defined. In model systems (5), classic tumor promoters induce the proliferation of initiated cells, leading to the progressive outgrowth of fully malignant cells. Such tumor promoters typically activate signal transduction pathways to stimulate cellular proliferation. In human breast cells, the overproduction of growth factors, or aberrant stimulation of growth factor receptors, may be responsible for the promotional phase of breast carcinogenesis (6).

Growth factors important for mammary epithelial cells, such as estrogen, EGF[4], TGF-α, and the IGFs, may all represent tumor promoters of human breast cancer. Thus, drugs which inhibit the ability of estrogen to activate the estrogen receptor (tamoxifen and other antiestrogens) and drugs which block growth factor receptors, such as antibodies specific for the Her2/neu receptor are now being used to treat or prevent breast cancer. However, inhibition of individual signal transduction pathways may be only partially effective, since multiple different signal transduction pathways can stimulate breast cell proliferation. It may be more effective to inhibit signal transduction at a more distal point in the cascade, where multiple mitogenic signals converge. Since transcription factors, the nuclear proteins which control DNA transcription and gene expression, are the most distal components of these converging mitogenic signal transduction

pathways, inhibitors of these transcription factors may be more potent inhibitors of breast cell growth.

A key family of transcription factors transducing multiple mitogenic signals is the AP-1 family. These transcription factors are complexes of DNA-binding proteins made up of dimers of Jun and Fos proteins, which bind DNA at specific AP-1 sites and regulate the transcription of AP-1-dependent genes. AP-1 transcription factors are expressed in most cell types, and are activated by specific kinases, such as the mitogen-activated and stress-activated kinases, which are themselves activated by diverse signals such as growth factor stimulation, exposure to light, oxidative stress, tumor promoters such as TPA, or oncogene overexpression or activation (7). Thus, AP-1 is a central component of many signal transduction pathways in many different cell types.

We have previously shown that normal breast cells have high basal levels of AP-1, while breast cancer cells have low basal levels. We hypothesized that the high basal level of AP-1 present in normal breast cells is required to support the growth of normal cells and that these cells would be more dependent on AP-1 for their growth than breast cancer cells. To investigate the role of AP-1 in controlling breast cell growth we have used normal breast cells, immortal breast cells, and breast cancer cells which are representative of breast cells at different stages of carcinogenesis. We have determined the effect of AP-1 blockade on the growth of these different breast cells using the cJun dominant-negative mutant, TAM-67. These studies demonstrate that normal and immortal human mammary epithelial cells, which have the highest basal AP-1 transcriptional activity, require AP-1 for their growth. Breast cancer cells which have relatively low basal AP-1 transcriptional activity are less sensitive to AP-1 blockade. Of the breast cancer cells tested, MCF7 cells were the most sensitive to the growth suppressive effects of the AP-1 inhibitor. MDA MB 435 cells showed slight sensitivity to the growth suppressive effects of TAM-67 when transiently transfected with the inhibitor. The other breast cancer cells tested, T47D and MDA MB 231, were resistant to the growth suppressive effects of AP-1 blockade. These results demonstrate that the growth of

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normal breast cells and some breast cancer cells is inhibited by AP-1 blockade, and suggest that AP-1 is a promising target for agents for the prevention or treatment of breast cancer.

MATERIALS AND METHODS:

Primary Cell Cultures and Cell Lines:

Human mammary epithelial cells and cell lines used in these studies are listed in Table 1. Cells used include normal HMECs isolated from epithelial organoids of human breast from Clonetics (passages 9-10); normal 184 cells provided by Dr. Martha Stampfer (8); nontumorigenic immortal cell lines derived from benzo(a)pyrene-treated 184 cells 184A1 and 184B5 (9), a nontumorigenic spontaneously immortalized HMEC cell line MCF10A (from Dr. J. Russo); and cancer cells: MCF7, a human breast adenocarcinoma cell line provided by Dr. Ken Cowan, and T47D, MDA MB 231, and MB MDA 435 (from ATCC). Cells were grown in the following culture media: MEGM (Clonetics, San Diego, CA) for normal HMECs 184, 184A1, and 184B5 (9, 10) DME/F-12 with 5% horse serum and supplements for MCF10A (11, 12) and Improved MEM (high zinc option; Life Technologies, Inc.) supplemented with 10% FCS and penicillin/streptomycin for the breast cancer cell lines. MCF-7 tTA cells were purchased from Clontech and MDA MB 435 rtTA cells were a gift from Dr. Doug Yee.

Transfection of Breast Cells:

The breast cells 184, HMEC-91, 184B5, MDA MB 231, MCF7, and T47-D were transfected using Fugene 6 reagent (Boehringer Mannheim); MCF10A and MDA MB 435 breast cells were transfected using the LT-1 transfection reagent (PanVera Corp.) according to manufacturer's recommendations.

Western Analysis:

Whole cell protein extracts normalized were electrophoresed on a 12% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad). Primary antibody used was rabbit anti-cJun Ab-1 from Oncogene Science (Cambridge, MA). Blots were developed using the enhanced chemiluminescence (ECL) procedure (Amersham).

Luciferase Assay to Measure AP-1 Activity:

AP-1 transcriptional activating activity in cells was measured using the enhanced luciferase assay (Tropix) as previously described (13). The cells were transfected with the Col-Z-Luc reporter gene containing the luciferase gene linked to 1100 bp of the human collagenase gene promoter which contains a single AP-1 binding site (TGAG/CTCA) between nucleotides -73 and -60. Transfected cells were lysed 36 hours after transfection and luciferase activity was measured with equal amounts of cell extract.

Cell Growth Assays:

Colony Formation Assay

2×10^5 cells were co-transfected in 35 mm wells with 0.5 ug pZeoSV (Invitrogen), and 5 ug of either pCMV (empty vector) or pCMV-TAM-67. Twelve hours after transfection the cells from each 35 mm well were split into four 35 mm wells. 24 hours after Zeocin (Invitrogen) was added to a final concentration of 400 ug/ml. After two weeks of selection in Zeocin, resistant colonies were stained with crystal violet and counted.

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Single cell proliferation assay:

Cells were co-transfected as described for the colony forming efficiency assay with 0.5 ug of pCMV-ß-gal and 5 ug of either pCMV (empty vector) or of pCMV-TAM-67. Twelve hours after transfection the cells were trypsinized and replated as single at cells densities of 0.2 to 1.0 X 10^5 in 100 mm plates. After approximately three doublings, colonies of cells were fixed and stained with X-Gal to detect cells expressing ß-galactosidase *in situ.* Colonies containing blue cells were visualized by light microscopy and scored for the number of blue cells per colony.

Cell proliferation assay of stably transfected Tet-on and Tet-off cell lines

The CellTiter 96^{TM} AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) was used to measure breast cancer cell growth according to the protocol provided by the manufacturer. 1000 to 2000 cells were seeded in a 96 well plate and doxycycline was added (MDA MB435 rtTA-vector or -TAM67 lines) or removed (MCF7 tTA-vector or -TAM67 lines) was added the next day and replaced every other day. A solution containing a 20:1 ratio of MTS and PMS was added to the cells for 2 hours at 37° C and absorption at 495 nm was determined. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Statistical Analyses:

Colony formation assays result in counts of numbers of colonies. After log-transformation, as indicated by Box-Cox analysis (14), TAM-67 transfected cells were compared to vector alone using two-sample t-tests. Single cell proliferation assays results in a distribution of cells per colony for each cell type. Wilcoxon rank sum tests were used to compare distributions between TAM-67 and vector transfected cells .

RESULTS:

Expression of the cJun dominant-negative mutant, TAM-67, in breast cells:

To determine whether AP-1 transcription factor complexes are involved in controlling breast cell growth we have investigated the effect of inhibiting AP-1 activity on breast cell proliferation. To block AP-1 activity we used the cJun dominant-negative mutant, TAM-67. TAM-67 is a mutant form of cJun in which the transactivation domain has been deleted leaving the DNA binding and the leucine zipper domains intact (Fig. 1A). We and others have used TAM-67 to investigate the role of AP-1 in cell transformation, cell differentiation, and apoptosis in many different cell types (13, 15-19) .

Expression of the TAM-67 protein in different breast cells was determined by immunoblot analysis of whole cell extracts of breast cells co-transfected with 0.5 ug of the pCMV-TAM-67 expression vector plus 0.1 ug of pCMV-ß-gal. The relative levels of TAM-67 protein expressed in the different cell lines was determined using volumes of protein extract adjusted for differences in transfection efficiencies using ß-galactosidase activity. TAM-67 protein was detected using antibodies directed against the DNA binding domain of the human cJun protein (Fig. 1B). Expression of the TAM-67 was observed in all cell lines transfected. These results demonstrate that in breast cells transiently transfected with pCMV-TAM-67, TAM-67 protein expression was lowest in MCF10A, was expressed at intermediate to high levels in the other breast cells tested (184, HMEC, 184B5, MCF-7, T47D, and MDA MB 435). While the expression level is low in MCF10A cells TAM-67 inhibits AP-1 activity in MCF10A cells and in all the other breast cells tested as shown below (Fig. 2A and 2B).

TAM-67 inhibits AP-1 activity in normal and malignant breast cells:

To determine whether TAM-67 inhibits AP-1 activity in the different breast cells, we cotransfected the luciferase reporter construct, Col-Z-Luc, with increasing amounts of the TAM-67 expression plasmid, pCMV-TAM-67. The results of these experiments are shown in Figure 2. We observed that the high basal AP-1 activity present in normal and immortal breast epithelial cell is significantly inhibited by TAM-67 expression (Figure 2A). Because basal AP-1 activity is relatively low in breast cancer cells (13), we determined the effect of TAM-67 expression on TPA-induced AP-1 transcriptional activity in the cancer cells (Fig. 2B). Increasing amounts pCMV-TAM-67 resulted in inhibition of TPA induced AP-1 activity in all breast cancer cell lines examined. In addition, we have observed that TAM-67 also inhibits serum-stimulated AP-1 activity in breast cancer cells (13, data not shown). These results demonstrate that TAM-67 inhibits AP-1-dependent activity in all of the different breast cells.

TAM-67 inhibits colony formation in immortal breast cells:

We next investigated whether inhibition of AP-1 transcriptional activity affects breast cell proliferation using a colony forming assay. This assay has been extensively used to demonstrate the effects of tumor suppressors and oncogenes on cell growth. As described in Materials and Methods, breast cells were co-transfected with pZeoSV and either pCMV vector or pCMV-TAM-67. The pZeoSV plasmid contains a zeocin resistance gene allowing selection of transfected cells. We have measured the effect of TAM-67 on colony formation in immortal and malignant breast cells. Normal mammary epithelial cells could not be analyzed because these cells undergo a finite number of doublings and did not form zeocin-resistant colonies. The colony forming results for all cell lines tested are shown in Figure 3. Colony formation of immortal breast cells was reduced when these cells were co-transfected with pCMV-TAM-67 compared to cells co-transfected with pCMV (Fig. 3B). The number of colonies were reduced by 62% and by 58% for MCF10A and 184B5, respectively. Colony formation of the MCF7 breast cancer cell line was reduced by 43% (Fig. 3C), that of MDA MB 435 cells was reduced by 17% (not statistically significant) while

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colony formation of T47D and MDA MB 231 breast cancer cells was not inhibited by expression of TAM-67 (Fig. 3C). Inhibition of AP-1 in T47D cells increased colony formation suggesting the possibility that AP-1 complexes in this particular cell line inhibit proliferation.

TAM-67 inhibits single cell proliferation:

To investigate the effect of AP-1 blockade on the growth of normal HMECs, as well as on the growth of immortal and malignant breast cancer cells, we used a second assay, the single cell proliferation assay (SCPA) previously described by Timchenko et al. (20). We used this assay to analyze normal, immortal, and breast cancer cell growth in the presence of TAM-67. The cells were co-transfected with 5 ug of the expression vector pCMV (empty vector) or pCMV-TAM-67 plus 0.5 ug of pCMV-ß-gal. After allowing recovery from the transfection the cells were plated at low cell densities and cultured to allow single cells to grow into small colonies ranging from 1-20 cells. The cells were then fixed and stained in situ for ß-galactosidase activity and transfected cells were identified as blue cells by light microscopy. The number of transfected cells observed per colony were scored and presented as a histogram of the percentage of colonies having 1, 2, 3, or more transfected cells per colony. An example of a blue colony obtained by transfection of MCF7 cells with pCMV-ß-gal is shown in Figure 4A.

Results from these experiments demonstrate that the number of ß-galactosidase expressing cells in TAM-67 transfected normal HMEC and immortal cell colonies are reduced as compared to vector-transfected cells (Fig. 4B). Both MCF7 and MDA MB 435 breast cancer cells also showed sensitivity to AP-1 blockade (Fig. 4C), while T47D and MDA MB 231 breast cancer cells were resistant (Fig. 4C). In the case of the sensitive breast cancer cells, MCF7 cells appear to be more sensitive than MDA MB 435 cells, as reflected by a larger decrease in the median number of blue cells induced by TAM-67 (8.1 blue cells/colony for pCMV transfected MCF7 compared to 2.8 blue cells/colony for pCMV-TAM-67 transfected MCF7, as compared to 3.0 blue cells/colony for pCMV transfected 435s compared to 2.0 blue cells /colony for pCMV-TAM-67 transfected 435s). The modest effect on MDA MB 435 cell growth seen in this assay is consistent with the slight (but

not significant) reduction of colonies seen in the colony forming assay (see Fig. 3C). We conclude from these results that TAM-67 expression inhibited the growth of normal and immortal breast cells, and also the breast cancer cell lines, MCF7, and MDA MB 435 cells (modestly sensitive to TAM-67), but that T47D and MDA MB 231 were resistant to AP-1 blockade.

Isolation of breast cancer clones expressing TAM-67 under the control of an inducible promoter:

To directly investigate the effect of AP-1 blockade on the growth of MCF7 and MDA MB 435 cells we created MCF7 and MDA MB 435 cell lines that express inducible TAM-67 protein. The Tet-off system was used for creation of MCF7 TAM-67 cell lines and the Tet-on system was used for the creation of MDA MB 435 TAM-67 cell lines. MCF7 tTA and MDA MB 435 rtTA cells were transfected with expression plasmids containing the flag-tagged TAM67 cDNA inserted downstream of a tetracycline-responsive transcriptional promoter and a hygromycin-selectable marker as described in Figure 5A. Hygromycin-resistant colonies were selected under conditions that repress expression of the TAM-67 cDNA. Hygromycin-resistant colonies were screened for inducible TAM-67 protein expression by immunoblotting with anti-cJun antibodies. Figure 5B shows the inducible expression of clones found to express TAM-67. TAM-67 protein expression was undetectable under uninduced conditions in both the TAM-67 transfectants of MCF7 Tet-off and MDA MB 435 Tet-on cell lines. However, after 48 hours of induction TAM-67 was expressed in all of the TAM-67 transfectants (Figure 5B), no TAM-67 was observed after induction in any vector transfectants (data not shown).

The functional activity of the inducible TAM-67 proteins was determined by analyzing inhibition of TPA-induced AP-1 transactivating activity (Figure 5C). Cells were transfected with the AP-1 reporter plasmid, Col-Z-Luc, which contains an AP-1-dependent TPA response element upstream of the luciferase gene. The cells were split 1:2 following transfection incubating half of the cells in medium containing doxycycline while incubating the other half of the cells in normal medium. After 36 hours of induction the cells were treated with TPA for 4-6 hours. Under these

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conditions (uninduced conditions) AP-1 activity is induced more than five fold in both MCF7 Tet-off and MDA MB 435 Tet-on cells. In contrast, under induced conditions when TAM-67 is expressed, TPA-induced AP-1 activity was reduced in MCF7 cells and in MDA MB 435 cells (Figure 5B). These results demonstrate that the TAM-67 protein induced in these two breast cell lines inhibits AP-1.

To determine whether AP-1 transactivating activity is required for anchorage dependent growth of breast cancer cells, we measured the growth of the cells under conditions in which TAM-67 was either repressed or induced (Figures 5D and 5E). As shown in Figure 5D, the growth of MCF7 Tet-off cells were inhibited in three independently isolated TAM-67 clones, while the growth of three vector-transfected clones was not inhibited. However MDA MB 435 TAM-67 clones grew as well as MDA MB 435 vector clones when TAM-67 protein was induced. These results suggest that expression of TAM-67 at these levels did not affect the growth of these MDA MB 435 clones. These results along with the results of studies of transiently-transfected cells, shown in Figs. 2-4, suggest that MCF7 cells are more sensitive than are MDA MB 435 cells to growth suppression by AP-1 blockade. Thus, normal breast cells are most sensitive, immortal breast cells and some breast cancer cells are moderately sensitive and other breast cancer cells (T47D and MDA MB 231) are resistant to the growth suppressive effects of the AP-1 inhibitor TAM-67.

DISCUSSION

The above results show that the growth of normal and immortal breast cells and some breast cancer cells is inhibited by AP-1 blockade, while the growth of several other breast cancer cell lines is not. We have previously shown that normal human breast cells express high basal levels of AP-1 activity and that breast cancer cells express relatively low levels of AP-1 activity (21) . The studies reported here suggest that these different levels of AP-1 in normal and malignant breast cells reflect a difference in these cells' dependence on AP-1 for their growth.

These findings indicate that normal and immortalized human breast cells are more dependent on AP-1 for the transduction of mitogenic signals than are breast cancer cells.

The present results demonstrating that premalignant breast cells depend on AP-1 to transduce mitogenic signals is consistent with previous reports demonstrating that normal human mammary epithelial cells require peptide growth factors to support their growth. Stampfer *et al.* (8) and Zajchowski *et al.* (22) have demonstrated that peptide growth factors are required for the *in vitro* growth of normal human mammary epithelial cells. In addition, Stampfer *et al.* (23) have shown that 184 normal human mammary epithelial cells, and the immortalized derivative 184B5, are both dependent on TGFα for continued growth demonstrating that normal breast cells require peptide growth factors to sustain their growth. The present results extend this observation to show that the activity of the AP-1 transcription factor, a downstream transducer of these peptide growth factors, is critical for the growth of normal and immortal breast cells.

Our results also demonstrate that breast cancer cells are less dependent on AP-1 for their growth than are normal breast cells. The breast cancer cell line most sensitive to AP-1 inhibition, MCF7, was more resistant than normal and immortal cells. The other cancer cell lines studied showed intermediate sensitivity (MDA MB 435) or resistance (T47D, MDA MB 231) to AP-1 blockade. In the case of MDA MB 435 breast cancer cells, we observed modest inhibition of growth in transiently transfected cells (Fig. 3 and 4) and no inhibition of growth in the Tet-on stable transfectants (Fig. 5D). This discrepancy may be due to higher levels of TAM-67 achieved in transiently transfected cells as compared to stably transfected cells. In addition, the present results suggest that at least for some of the breast cancer cell lines tested (MDA MB 231 and T47D), activation of AP-1 dependent pathways is not essential for their growth.

The breast cancer cell line T47D was observed to have an increased colony forming efficiency when AP-1 activity was blocked. However, this increase in growth was not observed using the single cell proliferation assay. This observation indicates that AP-1 activity may be involved in negative growth regulation of this breast cancer cell line. A role for AP-1 as a negative

regulator of cell growth has been reported for a number of cell lines. Studies are ongoing to investigate the role of AP-1 in regulation of the growth of T47D breast cancer cells.

It is possible that breast cancer cells that have genetic alterations in tumor suppressor genes, such as p53 or Rb mutations, or overexpression of oncogenes, such as c-erbB2/her2/neu, c-myc, or cyclin D, no longer require mitogenic signals that are normally transduced by AP-1. The observation that normal human breast cells, which lack these genetic alterations, require AP-1 activity for their growth is consistent with this hypothesis. Most of the breast cancer cells studied here have known genetic alterations (24-26). T47D, MDA MB 231, and MDA MB 435 have known p53 mutations, while MCF7 breast cancer cells, 184 normal breast cells, and the immortalized cells have been shown to express normal p53 (27). These genetic alterations may disrupt the mitogenic signal transduction pathway at a step distal to AP-1, and thus render these transformed cells more resistant to AP-1 inhibitors.

The results presented here demonstrate that the AP-1 transcription factor is an important mitogenic signaling complex for normal, immortal, and some breast cancer cells. These studies suggest that this transcription factor complex could be targeted for the development of future therapeutic agents. Thus, agents that inhibit AP-1 or that block AP-1 activation, such as inhibitors of Jun N-terminal kinases would be promising agents for the prevention or treatment of breast cancer.

ACKNOWLEDGMENTS

FIGURE LEGENDS

Figure 1. TAM-67, a dominant negative mutant of cJun.

A. Schematic representations of the protein functional domains in cJun and the cJun mutant, TAM-67. Three functional domains of cJun are shown: the transactivation domain, the DNA binding domain, and the dimerization domain. TAM-67 has most of the transactivation domain deleted.

B. Transient protein expression of TAM-67 in different breast cells. TAM-67 protein expression was determined 36 hours after cotransfection with 2.5 ug of the TAM-67 expression vector, pCMV-TAM-67, and 0.5 ug of pCMV-β-gal. Whole cell extracts were were normalized for transfection efficiency using β-galactosidase activity and separated by SDS-PAGE on 12% gels, transferred to nitrocellulose, probed with an anti-cJun antibody (Ab-1, Oncogene Science) and visualized using ECL (Amersham).

Figure 2. TAM-67 inhibition of Col-Z-Luc activity in different breast cells.

A. TAM-67 inhibition of basal Col-Z-Luc promoter activity in normal and immortal human breast cells. Cells were transfected with 1 ug of Col-Z-Luc reporter plasmid plus increasing amounts (0 ug, 0.1 ug or 0.5 ug) of the expression plasmid pCMV-TAM-67. pCMV (empty vector) was included (0.5 ug, 0.1 ug or 0 ug) to maintain equal amounts of DNA in each transfection. Transfection procedures were performed as described in "Experimental Procedures and Methods". Cells were lysed and luciferase activity was determined 36 hours after transfection.

B. TAM-67 inhibition of TPA induced Col-Z-Luc promoter activity in breast cancer cells.. Cells were transfected with 1 ug of Col-Z-Luc reporter plasmid plus increasing amounts pCMV-TAM-67 as described in (A). Induction of AP-1 activity was done 36 hours post-transfection by treating the cells with TPA (0.1 nM) for 4-6 hours.

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Figure 3. Colony Forming Efficiency of TAM-67 Transfected Breast Cells.

A. Colony formation of MCF7 cells transfected with vector or TAM-67. MCF7 cells cotransfected with pZeoSV and either pCMV (top) or pCMV-TAM-67 (bottom). Colonies formed after two weeks of selection are shown.

B and C. Colony formation of immortalized (B) and malignant (C) breast cells. Immortalized (B) and malignant (C) breast cells were transfected with pSVZeo (0.5 ug) and either pCMV (5ug) or pCMV-TAM67 (5ug). Transfections were performed as described in "Experimental Methods and Procedures". After two weeks of selection Zeocin resistant colonies were stained with crystal violet and counted. The data shows the average number of Zeocin resistant colonies from an experiments done in triplicate with error bars representing the SEM. The names of the different cell lines analyzed are given on the x-axis. * p-value<0.05.

Figure 4: Single Cell Proliferation Assay of Normal, Immortal, and Malignant Breast Cells.

A. Schematic diagram of the single cell proliferation assay. MCF7 cells were co-transfected with pCMV (5 ug) and pCMV-β-gal (0.5 ug) and stained with X-gal. A transfected colony (blue cells) and an untransfected colony (white cells) are shown.

B and C. Single cell proliferation assay of normal and immortal (B) and malignant (C) breast cells. The indicated breast cells were transfected with pCMV-β-gal (0.5 ug) and either pCMV (5 ug) or pCMV-TAM-67 (5 ug). After approximately three doublings the transfected cells were identified by staining *in situ* for β-galactosidase activity and the number of transfected cells per colony were counted. The results are shown as histograms of 1 to 20 cells per colony and were analyzed using the Wilcoxon rank sums test.

Fig. 5. Tet-off and Tet-on inducible protein expression systems

A. The Tet-off (top) and Tet-on (bottom) inducible protein expression systems are shown. The Tet-off system utilizes the tetracycline dependent transcriptional repression activity of the tTA protein. The TAM-67 cDNA was cloned downstream of a tTA repressible CMV promoter element (TetRE) in a plasmid having a hygromycin-selectable marker. The Tet-on system utilizes the tetracycline dependent transcriptional activation activity of the mutant transcription factor, rtTA. The TAM-67 cDNA was cloned downstream of a rtTA activatable CMV promoter element (TetRE) in a plasmid having a hygromycin-selectable marker. With the Tet-off system transfected cells are selected for hygromycin-resistance in the presence of doxycycline to maintain repression of TAM-67 expression and protein expression is induced by removal of doxycycline. With the Tet-on system transfected cells are selected for hygromycin-resistance in medium lacking doxycycline to maintain uninduced TAM-67 expression and protein expression is induced by addition of doxycycline.

B. TAM67 Protein Expression and AP-1 Activity in MCF7 Tet-off and MDA MB435 Tet-on cells. Immunodetection of induced TAM-67 protein expression in MCF7 Tet-off and MDA MB 435 Tet-on cells. Total cellular protein was extracted 48 hours after induction and equal amounts of protein was analyzed for TAM-67 expression using anti-cJun antibody. The TAM-67 protein band is indicated with an arrow.

C. Inhibition of TPA-induced AP-1 activity was determined by measuring the effect of TAM-67 protein induction on the activity of the transfected AP-1 dependent luciferase reporter plasmid, Col-Z-Luc. Cells were transfected with 1 ug of Col-Z-Luc for 12 hours and split into two plates, with or without doxycycline. After 36 hours of incubation the cells were treated with TPA for 4-6 hours at which time cell extracts were made and luciferase activity was measured. The data are presented as luciferase activity in the presence of TAM-67 relative to luciferase activity in the absence of TAM-67.

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D. Effect of induced TAM-67 expression on the growth of MCF7 and MDA MB 435 breast cancer cells. Growth of MCF7 Tet-off vector and TAM-67 clones and MDA MB435 Tet-on vector and TAM-67 clones was determined in the presence (filled squares) and absence (open squares) of doxycycline. Cell growth was measured using the MTS assay as described in "Experimental Methods and Procedures".

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A. THE DOMINANT NEGATIVE INHIBITOR OF AP-1
TAM-67
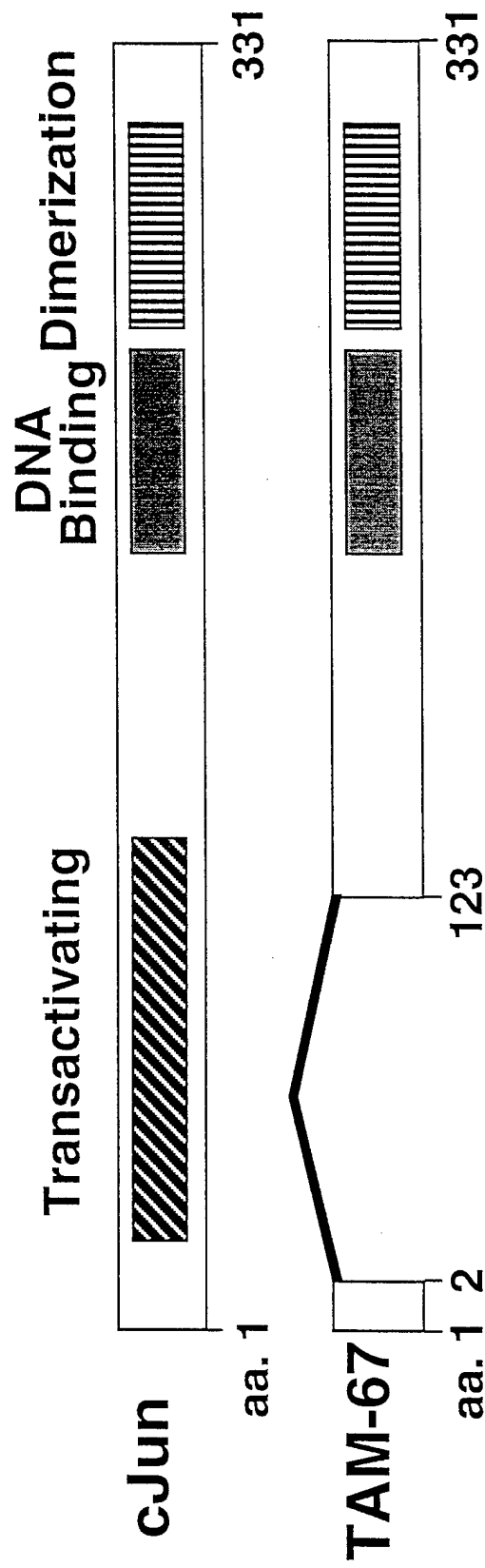
B.

MCF7
untransfected

MCF7

T47D

MDA MB 435

MDA MB 231

MCF10A

184B5

184

HMEC-91

[TAM67

A.

Relative Luciferase Activity (Percent)

Normal breast cells   Immortal breast cells

| ug TAM-67 DNA | 0 | .1 | .5 | 0 | .1 | .5 | | 0 | .1 | .5 | 0 | .1 | .5 |
| HMEC-91 | | | | 184 | | | | 184B5 | | | MCF10A | | |

B.

Breast cancer cells

Relative Luciferase activity (Percent)

ER +   ER-

ug TAM-67 DNA	0	.1	.5	0	.1	.5		0	.1	.5	0	.1	.5
TPA	+	+	+	+	+	+		+	+	+	+	+	+
	MCF7			T47D				MDA MB 231			MDA MB 435		

A.

pCMV

pCMV-TAM-67

B.

Number of Zeocin resistant colonies

20

15

10

5

0

184B5

MCF10A

*

*

■ pCMV

□ pCMV-TAM67

C.

Number of Zeocin resisitance colonies

100

80

60

40

20

0

MCF7

T47D

MDA MB 231

MDA MB 435

*

*

■ pCMV

□ pCMV-TAM67

A.

Co-transfect cells

TAM-67
expression
vector
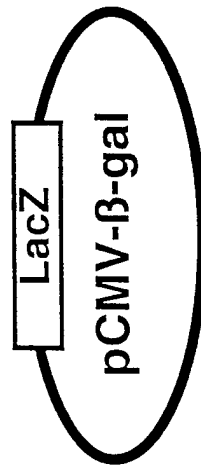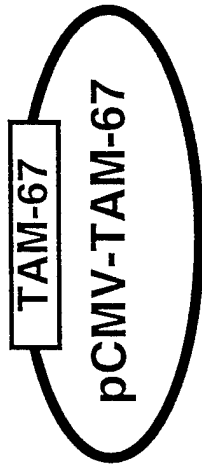
| TAM-67 |
pCMV-TAM-67

β-galactosidase
expression
vector

| LacZ |
pCMV-β-gal

Grow for 2-3 doublings

Fix and stain for
β-galactosidase activity.

Count the number of
blue cells per colony

Untransfected   transfected

B.

Normal breast cells | Immortal breast cells

HMEC-91   184   184B5   MCF10A

pCMV

pCMV-TAM-67
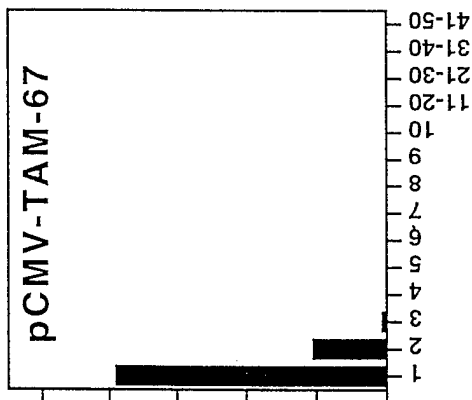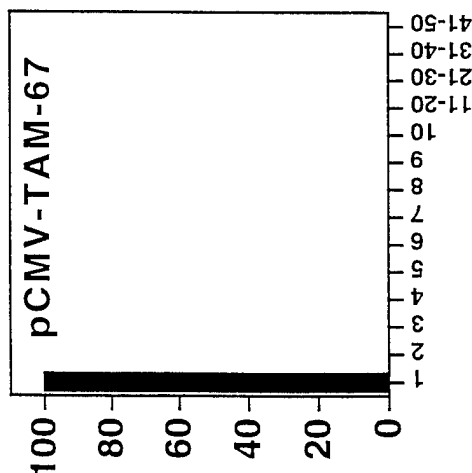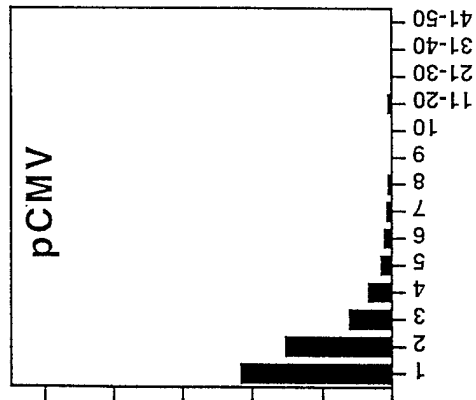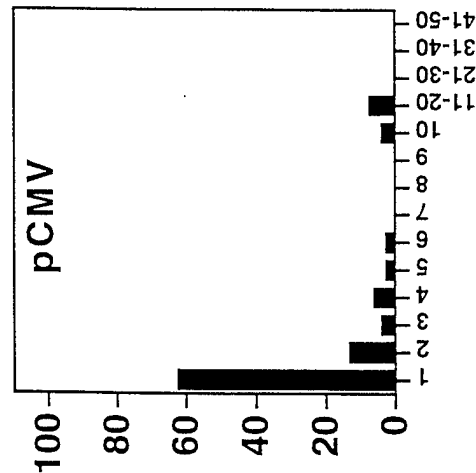
TAM-67<pCMV
p=0.07

TAM-67<pCMV
p=0.0001
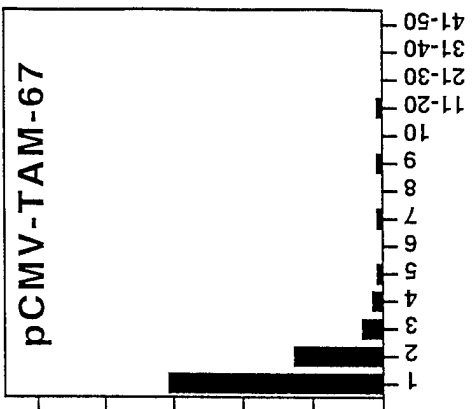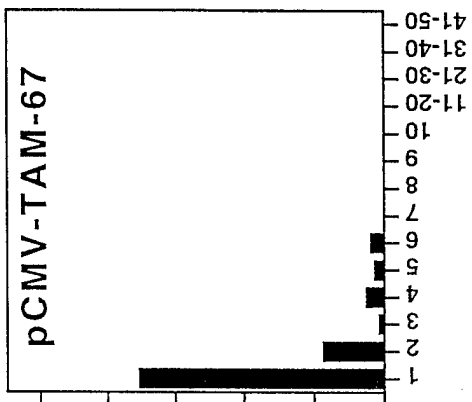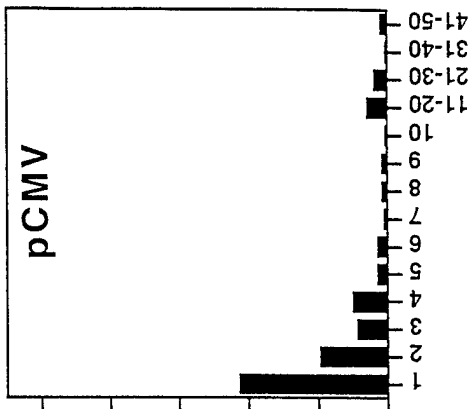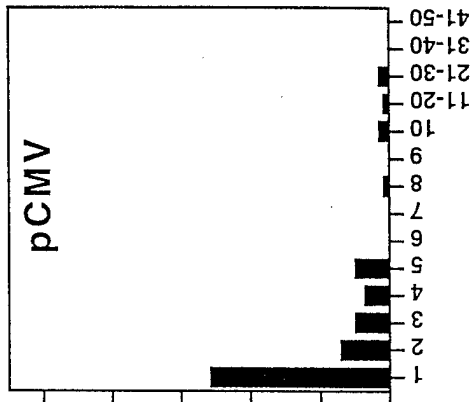
TAM-67<pCMV
p=0.005

TAM-67<pCMV
p=0.0002

%Blue Colonies

Number of Blue Cells/Colony

C.

ER+ breast cancer cells
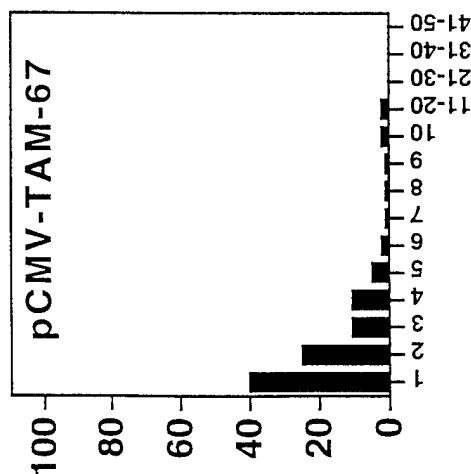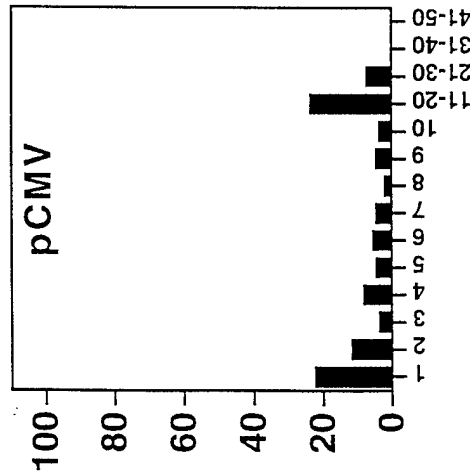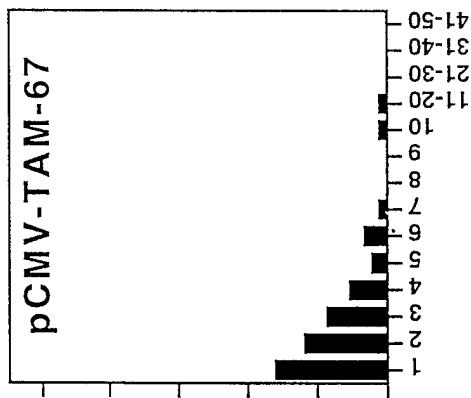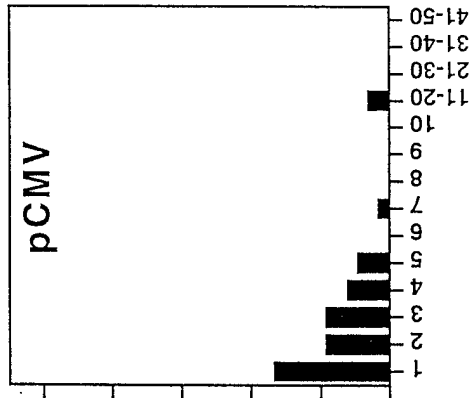
ER- breast cancer cells

% Blue Colonies

Number of Blue Cells/Colony

A.

In MCF7

TetRE
"Tet-Off"
- Tet

TetRE
"Tet-Off"
- Tet

In MDA MB 435

TetRE
"Tet-On"
- Tet

TetRE
"Tet-On"
+ Tet
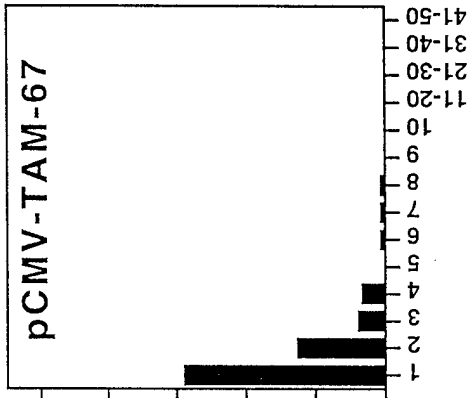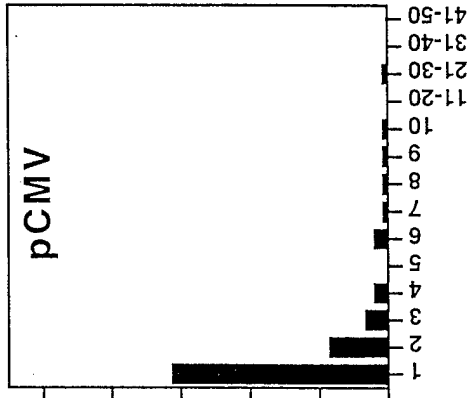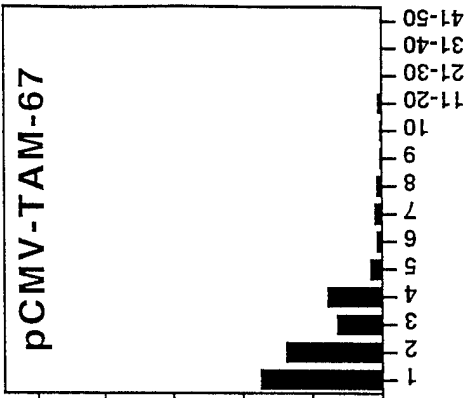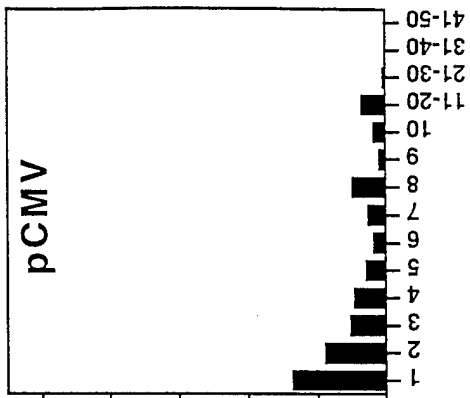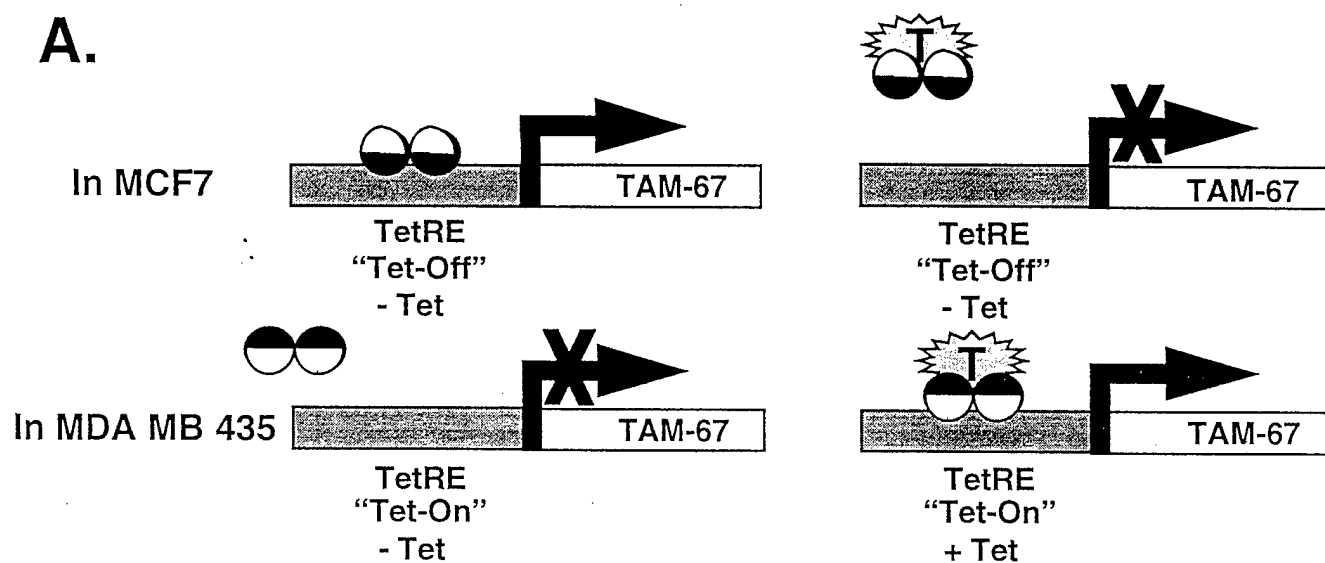
= Tetracycline

= tTA

= rtTA

B.

Dox

MCF-7 Tet-off

− + − + − +
#62 #67 #72

MDA MB435 Tet-on

− + − +
#33 #36

← TAM-67
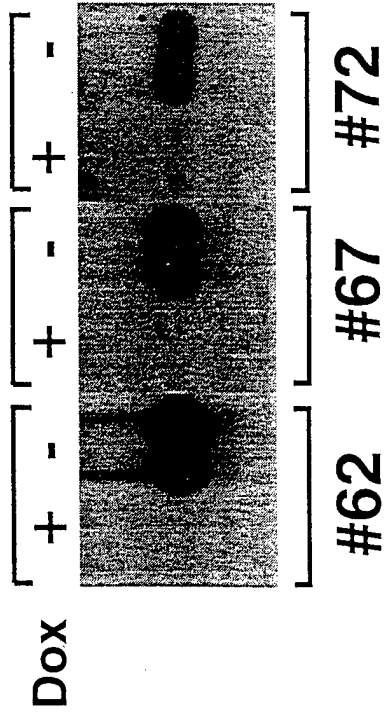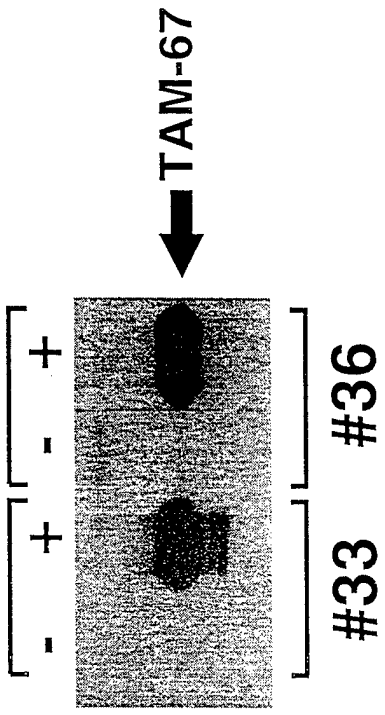
C.
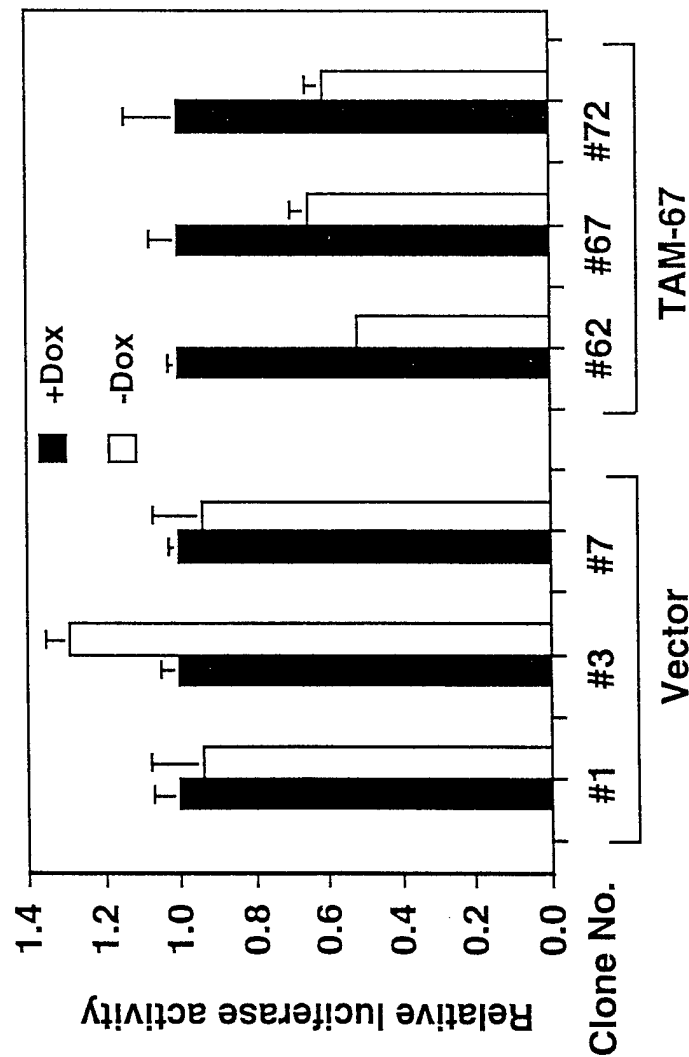
MCF7 Tet-Off clones

MDA MB 435 Tet-On clones

MCF7 tet-off clones

D.

Vector clone #1

Vector clone #3

Vector clone #7

TAM-67 clone #62

TAM-67 clone #67

TAM-67 clone #72

+DOX
-DOX

+Dox
-Dox

Relative Cell Growth
(O.D. 490 nm)

Days of growth

E. MDA MB 435 tet-on clones

Vector clone #3

Vector clone #1

TAM-67 clone #36

TAM-67 clone #33

Relative Cell Growth (O.D. 495 nm)

Days of growth